121,017

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :	A1	(11) International Publication Number: WO 91/16335
C07K 3/12, 3/28		(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US91/02766

(22) International Filing Date: 25 April 1991 (25.04.91)

(30) Priority data:
515,474   26 April 1990 (26.04.90)   US

(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventors: NASCIMENTO, Carlos, George ; 1907 South Forest Hill, Danville, CA 94526 (US). CALDERON-CACIA, Maria, Dolores ; 245800 Industrial Boulevard, No. S2241, Hayward, CA 94545 (US).

(74) Agents: ROBINS, Roberta, L. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: GLYCOSYLATED PDGF

(57) Abstract

Hyperglycosylated PDGF and methods of producing and isolating the same are disclosed. The hyperglycosylated PDGF displays markedly increased activity when compared to a less or nonglycosylated counterpart. The hyperglycosylated PDGF can be produced recombinantly in yeast and isolated from the less glycosylated fraction using lectin affinity chromatography.

5   GLYCOSYLATED PDGF

                           Description

       Technical Field
10   The instant invention relates generally to
       PDGF. More particularly, the present invention relates
       to a hyperglycosylated PDGF with increased biological
       activity, and methods of making and isolating the same.

15   Background
            Platelet Derived Growth Factor (PDGF) is the
       major mitogen in serum for mesenchymal-derived cells.
       PDGF promotes cell division of a variety of cells
       including smooth muscle cells, fibroblasts, and glial
20   cells. PDGF is stored in platelet alpha-granules and is
       released locally during platelet activation when blood
       vessels are injured. It is also a potent chemoattractant
       for monocytes, neutrophils, fibroblasts, and smooth
       muscle cells. These activities make PDGF an important
25   component in tissue repair processes.
            Native PDGF consists of dimers of homologous
       polypeptide chains, denoted A and B. Johnsson et al.,
       Biochem Biophys Res Commun (1982) 104:66-74. The mature
       A and B monomers exhibit nearly 60% homology. Whether
30   native PDGF is a heterodimer or a mixture of homodimers
       is not known, however, the dimeric structure is
       functionally important, since reduction of the disulfide
       bonds irreversibly destroys the biological activity of
       PDGF.

35

The complete primary structure of th PDGF
A-chain precursor has been deduced from its complementary
DNA sequence. Betsholtz et al., Nature (1986) 320:695-
699. Furthermore, Heldin et al., Nature (1986),
5   describes an osteosarcoma-derived growth factor (ODGF)
that is structurally related to putative PDGF A-chain
homodimer and studies on ODGF suggest that the PDGF A-
chain homodimer would exhibit biological activity.

          Similarly, the amino acid sequence of the
10   B-chain precursor has been described. Collins et al.,
Nature (1985) 316:748-750. The B-chain is derived by
proteolytic processing of this precursor which is encoded
by the c-sis gene, the cellular counterpart to the
transforming gene v-sis of simian sarcoma virus (SSV).
15   The SSV v-sis gene encodes a protein called p28sis, with
a molecular weight under nonreducing conditions of about
24 kDa (Robbins et al., Nature (1983) 305:605-608).
p28sis is highly homologous (approximately 96%) to the
PDGF B-chain. Mitogenic activity of p28sis and the
20   B-chain homologous region has been demonstrated in yeast
expression products. Kelly et al., EMBO J (1985) 4:3399-
3405.

          PDGF and analogs thereof have been expressed in
mammalian cell systems. See, e.g., King et al., Proc
25   Natl Acad Sci USA (1985) 82:5295-5299; Clarke et al.,
Nature (1984) 308:464-467; Gazit et al., Cell (1984)
39:89-97; and Josephs et al., Science (1984) 225:636-
639. Additionally, Kelly et al., EMBO J (1985) 4:3399-
3405, and U.S. patent nos. 4,766,073, 4,769,328 and
30   4,889,919 disclose methods for expressing PDGF A- and B-
chain analogs in yeast, and homodimers and heterodimers
thereof. European Patent Application 88302116.4
describes methods for purification of recombinant PDGF
B-chain and U.S. patent no. 4,479,896 describes the
35   recovery of PDGF peptides from human platelets.

Deule et al., J Biol Chem (1981) 256:8896-
8899, found that PDGF A and B, isolated from human
platelets, are glycoproteins. Hannick et al., Mol Cell
Biol (1986) 6:1343-1348, localized the v-sis gene product
5   to the endoplasmic reticulum-Golgi compartment and
postulated that the protein was glycosylated, based on a
reduction of molecular weight thereof after treatment
with a glycosylation inhibitor or a deglycosylating
agent. Klein et al., Virology (1988) 64:403-410, found
10   that cells transformed with SSV release a highly
glycosylated molecule termed gp200sis, in addition to
p28sis, which is recognized by anti-PDGF serum and
appears to act as a PDGF-like growth factor. However,
none of the above-described references disclose the
15   isolation of a hyperglycosylated PDGF molecule displaying
increased biological activity.

Glycoproteins produced in eucaryots contain
carbohydrate chains linked to Asn amino acid residues, in
the case of N-linked oligosaccharides, and Ser or Thr
20   residues, in the case of O-linked sugars. Additionally,
the carbohydrate chains found on glycoproteins produced
in yeast consist primarily of mannose moieties. Certain
yeast species also add terminal galactose and N-
acetylglucosamine residues to the sugar molecule. PDGF
25   A-chain contains both putative N- and O-glycosylation
sites while the PDGF B-chain includes several possible
O-linked glycosylation sites.

Disclosure of the Invention
30   The present invention is based on the
unexpected discovery of a hyperglycosylated PDGF B
homodimer which, when isolated from a heterogenous
mixture, displays dramatically increased biological
activity.
35

-4-

Accordingly, in one embodiment, the instant invention is directed to hyperglycosylated PDGF.

In another embodiment, the invention is directed to a method of producing hyperglycosylated PDGF
5   comprising:

(a) providing a mixture containing a hypergly-cosylated PDGF fraction and a less glycosylated PDGF fraction; and

(b) isolating the hyperglycosylated PDGF from
10 the mixture.

In particularly preferred embodiments, the hyperglycosylated PDGF is PDGF B-B homodimer having at least 17 moles of mannose per mole of dimer and the hyperglycosylated B-B homodimer is isolated from the less
15 glycosylated fraction using lectin affinity chromatography.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.
20

Brief Description of the Figures

Figure 1 shows the nucleotide sequence and corresponding amino acid sequence for one form of the PDGF A-chain polypeptide (designated 13-1).
25   Figure 2 shows the nucleotide sequence and corresponding amino acid sequence for a second form of the PDGF A-chain polypeptide (designated D1).

Figure 3 depicts the nucleotide sequence and corresponding amino acid sequence of a PDGF B-chain
30 polypeptide.

Figure 4 shows the nucleotide sequence and deduced amino acid sequence of the 13-1 A-chain precursor. The boxed portion designates the mature polypeptide.
35

Figure 5 depicts the nucleotide sequence and deduced amino acid sequence of the D1 A-chain precursor. The boxed portion designates the mature polypeptide.

Figure 6 is a diagram of plasmid pYAGL7PB, described in the examples.

Figure 7 is a diagram of plasmid pYpA6, described in the examples.

Figure 8 is a diagram of plasmid pYpA134, described in the examples.

Figure 9 is a diagram of plasmid pAB24, described in the examples.

Figure 10 is a graph showing the effect of the degree of glycosylation of PDGF B-B homodimer (determined by ConA affinity chromatography) on mitogenic activity.

Figure 11 is a typical chromatogram obtained when hyperglycosylated PDGF and less glycosylated PDGF are separated using ConA affinity chromatography.

Figure 12 shows the in vivo incorporation of ³H-mannose into PDGF B-producing cells.

Figure 13 is the nucleotide sequence of the SV40 region used to make plasmid pSV7d, described in the examples.

Figure 14 is a map of the plasmid pSV7d, described in the examples.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of protein chemistry, molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Scopes, R.K., Protein Purification Principles and Practice, 2nd ed. (Springer-Verlag 1987); Methods in Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.);

-6-

Maniatis, Fritsch & Sambrook, <u>Molecular Cloning: A</u>
<u>Laboratory Manual</u> (1982); <u>Oligonucleotide Synthesis</u> (M.J.
Gait ed. 1984); and <u>Handbook of Experimental Immunology</u>,
Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986,

5   Blackwell Scientific Publications).

All patents, patent applications, and publi-
cations mentioned herein, whether supra or infra, are
hereby incorporated by reference.

10   A. <u>Definitions</u>

In describing the present invention, the
following terms will be employed, and are intended to be
defined as indicated below.

As used herein, the term "PDGF" refers to any

15   active form of PDGF (as determined by the assays
described in the Experimental section). Thus, the PDGF
molecule may be either an A-A, B-B, or A-B dimer, or
fusion proteins thereof, the individual chains having a
primary sequence as depicted in Figures 1, 2, or 3 and

20   analogs of these amino acid sequences which are
substantially homologous and functionally equivalent
thereto. The term "substantially homologous" intends
that the number of amino acid variations (including
substitutions, additions and/or deletions) in the

25   sequence be less than about 10, preferably less than
about 3. The term "functionally equivalent" refers to
sequences of an analog which define a chain that will
produce a protein having the biological activity of PDGF
(as measured by the assay described in Example 5). Thus,

30   the A- and B-chain amino acid sequences need not be
identical to the depicted sequences.

"A composition of hyperglycosylated" or "highly
glycosylated" PDGF as used herein refers to an active
composition comprising at least an average of 10 moles of

35   carbohydrate per mole of dimer, more preferably an

average of 12-15 moles of carbohydrate per mole of dimer, and most pref rably an average of 17 moles of carbohydrate per mole of dimer. The composition can include either nonglycosylated or less glycosylated PDGF

5   forms so long as the average amount of carbohydrate per mole of dimer is as stated above. The carbohydrate present on the hyperglycosylated PDGF can be any carbohydrate that will bind the molecule and that does not destroy the biological activity of the

10   hyperglycosylated PDGF composition. Examples of sugar moieties useful in the instant invention are described more fully below. These sugars can be added as monomeric units or as oligomers, as described below. "Less glycosylated" PDGF is a PDGF molecule having less carbo-

15   hydrate moieties bound thereto than the hyperglycosylated PDGF described above.

A composition containing A is "substantially free of" B when at least 85% by weight of the total A+B in the composition is A. Preferably, A comprises at

20   least about 90% by weight of the total of A+B in the composition, more preferably at least about 95% or even 99% by weight.

The term "recombinant" as used herein to characterize DNA encoding PDGF A- or B-chain polypeptides

25   intends DNA of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is either a DNA sequence not occurring in nature or a DNA sequence linked to DNA other than that to which it is linked in nature.

30   A "replicon" is any genetic element (e.g., a plasmid, a chromosome, a virus) that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

A "vector" is a replicon in which another

35   polynucleotide segment is attached, so as to bring about

the replication and/or expression of the attached
segment. An "expression vector" refers to a vector
capable of autonomous replication or integration and
contains control sequences which direct the transcription
5   and translation of the PDGF A- or B-chain DNA in an
appropriate host.

A "coding sequence" is a polynucleotide
sequence which is transcribed and/or translated into a
polypeptide.

10   A "promoter sequence" is a DNA regulatory
region capable of binding RNA polymerase and initiating
transcription of a downstream (i.e., in the 3' direction)
coding sequence.

A coding sequence is "under the control" of the
15   promoter sequence in a cell when transcription of the
coding sequence results from the binding of RNA
polymerase to the promoter sequence; translation of the
resulting mRNA then results in the polypeptide encoded
within the coding sequence.

20   "Operably linked" refers to a juxtaposition
wherein the components are configured so as to perform
their usual function. Thus, control sequences operably
linked to a coding sequence are capable of effecting the
expression of the coding sequence.

25   "Control sequences" refers to those sequences
which control the transcription and/or translation of the
coding sequence(s); these may include, but are not
limited to, promoter sequences, transcriptional
initiation and termination sequences, and translational
30   initiation and termination sequences, and translational
initiation and termination sequences. In addition,
"control sequences" refers to sequences which control the
processing of the polypeptide encoded within the coding
sequence; these may include, but are not limited to
35

sequences controlling secretion, protease cleavage, and
glycosylation of the polypeptide.

A "signal sequence" can be included before the
coding sequence. This sequence encodes a signal peptide,
5   N-terminal to the polypeptide, that communicates to the
host cell to direct the polypeptide to the cell surface
or secrete the polypeptide into the media, and this
signal peptide is clipped off by the host cell before the
protein leaves the cell. Signal sequences can be found
10 associated with a variety of proteins native to
procaryots and eucaryots. For instance, alpha-factor, a
native yeast protein, is secreted from yeast, and its
signal sequence can be attached to heterologous proteins
to be secreted into the media (See U.S. Patent 4,546,082,
15 EPO 0 116 201, publication date 12 January 1983; U.S.
Patent Application Ser. No. 522,909, filed 12 August
1983). Further, the alpha-factor and its analogs have
been found to secrete heterologous proteins from a
variety of yeast, such as Saccharomyces and
20 Kluyveromyces, (EPO 88312306.9 filed 23 December 1988;
U.S. Patent Application Ser. No. 139,682, filed 30
December 1987, and EPO Pub. No. 0 301 669, publication
date 1 February 1989).

"Transformation" is the insertion of an
25 exogenous polynucleotide into a host cell. The exogenous
polynucleotide may be maintained as a plasmid, or
alternatively, may be integrated within the host genome.

B.   General Methods
30   Central to the instant invention is the
discovery that PDGF, isolated from eucaryotic cells,
exists as a heterogenous mixture of hyperglycosylated and
less glycosylated forms, as defined above. These
glycoproteins can be separated and the resulting
35 hyperglycosylated population exhibits surprisingly

greater mitogenic activity than the less glycosylated
PDGF.

PDGF A-A, B-B or A-B dimers can be isolated or
produced recombinantly in suitable cell expression
5   systems, under conditions favoring glycosylation,
according to the protocols described herein. Methods for
producing recombinant PDGF are also explained in commonly
owned, copending U.S. Patent Application ser. no.
041,299, filed 4 April 1987, the disclosure of which is
10   incorporated herein by reference in its entirety. Highly
glycosylated fractions are separated from less
glycosylated proteins by the method described below to
yield a hyperglcosylated PDGF fraction. Alternatively,
PDGF dimers can be synthetically produced, using protein
15   synthesis techniques well known in the art and explained
in more detail below.

Production of Recombinant Hyperglycosylated PDGF

20   1.   Recombinant PDGF DNA

The recombinant PDGF of the invention consists
of highly glycosylated A-A, B-B, or A-B dimers. Two
amino acid sequences for the PDGF A-chain are shown in
Figures 1 and 2, respectively. Figure 3 depicts an amino
25   acid sequence for the PDGF B-chain. The invention also
encompasses analogs of these amino acid sequences which
are substantially homologous and functionally equivalent
thereto, as defined above. DNA sequences for use in
eucaryotic expression systems, encoding the PDGF A-chain,
30   are shown in Figures 1 and 2, respectively, while that
for the PDGF B-chain is shown in Figure 3.

The recombinant PDGF A- or B-chain DNA may be
genomic, cDNA or synthetic DNA. By way of example, DNA
encoding the PDGF A- or B-chain can be obtained from a
35   cDNA library prepared from mRNA of a PDGF-producing cell

line. The·library can be probed with oligonucleotide
sequences based on the sequences disclosed in the
figures. Clones that hybridize with the probes can be
used to probe human genomic libraries to obtain analogous

5   genomic DNA encoding the PDGF polypeptides. Based on the
amino acid sequences illustrated, synthetic genes
encoding the PDGF A- and B-chain may be prepared _in vitro_
by synthesizing individual overlapping complementary
oligonucleotides and filling in single stranded

10   nonoverlapping portions using DNA polymerase in the
presence of the deoxyribonucleotide triphosphates.

     2. Cloning PDGF DNA
             The PDGF A- and B-chain DNA can be cloned into

15   any suitable replicon to create a vector, and thereby be
maintained in a composition which is substantially free
of vectors that do not contain the PDGF gene of interest
(e.g., other clones derived from the library). Numerous
cloning vectors are known to those of skill in the art,

20   and the selection of an appropriate cloning vector is a
matter of choice. However, preferred are those vectors
for expression in eucaryotic cells, most preferably yeast
cells, since these systems provide glycosylated products.
The PDGF products of the instant invention can also be

25   produced in bacteria and later glycosylated with any
suitable sugar moieties, using techniques known in the
art, and described in more detail below.
             Numerous cloning vectors are known to those of
skill in the art and the selection of an appropriate

30   cloning vector and host cells for use therewith is a
matter of choice. Examples of vectors for cloning and
host cells which they transform (in parenthesis) include
the bacteriophage lambda (E. coli); pBR322 (E. coli);
pACYC 177 (E. coli); pKT 230, pGV1106, and pLAFR1 (all

35   useful in gram-negative bacteria); pME290 (non-E. coli

gram-negative bacteria), pHV 14 (E. coli and Bacillus
subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6
(Streptomyces), actin phage C31 (Streptomyces), YIp5
(Saccharomyces), YCp19 (Saccharomyces), and bovine
papilloma virus (mammalian cells).

3.   Expression of PDGF DNA

          The polynucleotide sequence encoding the PDGF
A- or B-chain polypeptides are expressed by inserting the
sequence into an appropriate replicon thereby creating an
expression vector, and introducing the resulting
expression vector into a compatible host.

          In creating an expression vector the sequence
encoding the PDGF A- or B-chain polypeptide is located in
the vector with the appropriate control sequences. The
positioning and orientation of the coding sequence with
respect to the control sequences is such that the coding
sequence is transcribed under the control of the control
sequences: i.e., the promoter will control the
transcription of the mRNA derived from the coding
sequence; and the ribosomes will bind at the ribosomal
binding site to begin the translational process; and the
stop codon used to terminate translation will be upstream
from the transcriptional termination codon. Commonly
used procaryotic control sequences include such commonly
used promoters as the ß-lactamase (penicillinase) and
lactose (lac) promoter systems (Chang et al., Nature
(1977) 198:1056) and the tryptophan (trp) promoter system
(Goeddel et al., Nucleic Acids Res (1980) 8:4057) and the
lambda-derived P_L promoter and N-gene ribosome binding
site (Shimatake et al., Nature (1981) 292:128). Control
sequences for yeast vectors include promoters for the
synthesis of glycolytic enzymes (Hess et al., J Adv
Enzyme Reg (1968) 7:149; Holland et al., Biochemistry
(1978) 17:4900). Additional promoters known in the art

include the promoter for 3-phosphoglycerat kinase
(Hitzeman et al., J Biol Chem (1980) 255:2073). Other
promoters, which have the additional advantage of
transcription controlled by growth conditions and/or
genetic background are the promoter regions for alcohol
dehydrogenase 2 (ADH2), isocytochrome C, acid
phosphatase, degradative enzymes associated with nitrogen
metabolism, the α-factor system and enzymes responsible
for maltose and galactose utilization. It is also
believed that terminator sequences are desirable at the
3' end of the coding sequences. Such terminators are
found in the 3' untranslated region following the coding
sequences in yeast-derived genes. Expression vectors for
mammalian cells such as VERO, HeLa or CHO cells
ordinarily include promoters and control sequences
compatible with such cells as, for example, the commonly
used early and late promoters from Simian Virus 40 (SV40)
(Fiers et al., Nature (1978) 273:113), or other viral
promoters such as those derived from polyoma, Adenovirus
2, bovine papilloma virus, or avian sarcoma viruses. The
controllable promoter, hMTII (Karin, M., et al., Nature
(1982) 299:797-802) may also be used.

In addition to control sequences, it may be
desirable to add regulatory sequences which allow for
regulation of the expression of the PDGF A- or B-chain
gene relative to the growth of the host cell. Examples
of regulatory systems are those which cause the
expression of a gene to be turned on or off in response
to a chemical or physical stimulus, including the
presence of a regulatory compound. In procaryotic
systems these would include the lac and trp operator
systems. In eucaryotic systems induction can occur in
metallothionein genes with heavy metals and the mouse
mammary tumor virus (MMTV) system with steroids. In
these cases, the sequence encoding the PDGF A-chain

polypeptides would be placed in tandem with the regulatory element.

There are also selective elements which give rise to DNA amplification which in turn can result in higher levels of specific protein production. In higher eucaryotic systems these include the dihydrofolate reductase gene (dhfr) which is amplified in the presence of methotrexate, and adenosine deaminase (ADA) in the presence of deoxycorfomycin. In other eucaryotic systems, such as yeast, the uracil gene (ura) and/or the leucine gene (leu) can be used as selectable markers. For amplification in such systems, copper and tunicamycin resistance genes can be included in the vector and amplification can be achieved by the inclusion of copper and tunicamycin in the expression medium. In these cases the sequence encoding the PDGF A- or B-chain polypeptides may either be present on the same plasmid or merely be cotransfected together with the selectable element to allow for integration within the host cell genome near each other.

Other types of regulatory elements may also be present in the vector, i.e., those which are not necessarily in tandem with the sequence encoding PDGF A- or B-chain. An example is the SV40 enhancer sequence, which, by its mere presence, causes an enhancement of expression of genes distal to it.

Modification of the sequence encoding PDGF A- or B-chain, prior to its insertion into the replicon, may be desirable or necessary, depending upon the expression system chosen, For example, in some cases, it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation, i.e., to maintain the reading frame. It may also be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with

subsequent cleavage of the secretory signal. The
techniques for modifying nucle tide sequences utilizing
cloning are well known to th se skilled in the art. They
include, e.g., the use of restriction enzymes, of enzymes

5   such as Bal31, to remove excess nucleotides, and of
chemically synthesized oligonucleotides for use as
adapters, to replace lost nucleotides, and in site-
directed mutagenesis.

     The appropriately modified sequence encoding

10   the PDGF A- or B-chain polypeptide may be ligated to the
control sequences prior to insertion into a vector.
Alternatively, the coding sequence can be cloned directly
into an expression vector which already contains the
control sequences and an appropriate restriction site.

15   For expression of the PDGF A- or B-chain polypeptide in
procaryots and in yeast, the control sequences will
necessarily be heterologous to the coding sequence. In
cases where the PDGF A- or B-chain gene is to be
expressed in cell lines derived from vertebrates, the

20   control sequences may be either heterologous or
homologous, depending upon the particular cell line.

     Depending on the host cell used, transformation
is done using standard techniques appropriate to such
cells. The calcium treatment employing calcium chloride,

25   as described by Cohen, S.N., Proc Natl Acad Sci (USA)
(1972) 69:2110, or the RbCl$_2$ method described in Maniatis
et al., Molecular Cloning: A Laboratory Manual (1982)
Cold Spring Harbor Press, p. 254, and Hanahan, D., J Mol
Biol (1983) 166:557-580, may be used for procaryots or

30   other cells which contain substantial cell wall barriers.
For mammalian cells without such cell walls, the calcium
phosphate precipitation method of Graham and van der Eb,
Virology (1978) 52:546, optionally as modified by Wigler,
M., et al., Cell (1979) 16:777-785, may be used.

35   Transformations into yeast may be carried out according

-16-

to the method of Beggs, J.D., _Nature_ (1978) _275_:104-109,
or of Hinnen, A., et al., _Proc Natl Acad Sci (USA)_ (1978)
_75_:1929.

5   Other systems for expression of eucaryotic or
viral genomes include insect cells and vectors suitable
for use in these cells. These systems are known in the
art, and include, for example, insect expression transfer
vectors derived from the baculovirus _Autographa_
californica nuclear polyhedrosis virus (AcNPV), which is
10   a helper-independent, viral expression vector.
Expression vectors derived from this system usually use
the strong viral polyhedrin gene promoter to drive
expression of heterologous genes. Methods for the
introduction of heterologous DNA into the desired site in
15   the baculovirus are known in the art. (See Summer and
Smith, Texas Agricultural Experiment Station Bulletin No.
1555; Smith et al., _Mol and Cell Biol_ (1983) _3_:2156-
2165; and Luckow and Sumners, _Virology_ (1989) _17_:31).
For example, the insertion can be into a gene such as the
20   polyhedrin gene, by homologous recombination; insertion
can also be into a restriction enzyme site engineered
into the desired baculovirus gene. Sequences encoding
signal peptides can also be used in these expression
systems since these peptides are recognized by insect
25   cells and will cause the secretion of the expressed
product into the expression medium.
          Transformed cells are then grown under
conditions which permit expression of the PDGF A- or
B-chain gene and assembly of the expression product into
30   a biologically active PDGF (i.e., into a dimeric form).
In addition to producing recombinant PDGF A-chain or
B-chain homodimers, the present invention permits
production of heterodimers of PDGF A-chain and PDGF
B-chain by co-expressing the genes for both PDGF A-chain
35   and PDGF B-chain through use of separate vectors or a

single vector than c ntains an A-chain gene and the
B-chain gene. The recombinant PDGF protein thus
synthesized is then isolated from the host cells and
purified. If the expression system secretes the PDGF
5   into the growth media, the PDGF is isolated directly from
the media. If the recombinant PDGF is not secreted, it
is isolated from cell lysates. The selection of the
appropriate growth conditions and recovery methods are
within the skill of the art. With regard to
10 purification, see for instance EPA Publication No.
0177957 and Nature (1986) 319:511-514.
It has been found that PDGF isolated from yeast
expression systems exists in a heterogenous state. That
is, both a hyperglycosylated fraction and a less glycosy-
15 lated fraction were found in expression products from
yeast. These fractions exhibit differing biological
activity, as determined by the mitogenic assay described
herein--the more highly glycosylated fraction and
mixtures containing a higher percentage of the highly
20 glycosylated fraction, being more active. The two
fractions can be further separated, as described below,
to yield a purer form of hyperglycosylated PDGF.
If the PDGF has been expressed in systems which
produce nonglycosylated products, i.e. in bacteria, the
25 proteins can be glycosylated by the addition of oligosac-
charide units to appropriate glycosylation sites. It is
known that O-glycoproteins are formed by linkages between
Ser and Thr with a variety of sugars including galactose,
xylose, mannose, and fructose, among others. Montreuil,
30 J., Advances in Carbohydrate Chemistry and Biochemistry,
Vol. 37, pp. 157-223 (Academic Press 1980). Other sugars
that will find use with the instant invention include but
are not limited to aldoses and ketoses such as glyceral-
dehyde, erythrose, ribose, allose, altrose, arabinose,
35 glucose, threose, gulose, idose, lyxose, talose, and

derivatives thereof such as glycosides, glycosylamines,
O-acyl derivatives, O-methyl derivatives, amino sugars,
among others. These sugars can be present as mono-, di-,
or polysaccharides.

5   N-linked glycosylation typically occurs between
a sugar and an Asn residue at locations in a peptide
chain having the amino acid sequence Asn-X-Ser(or Thr),
where X can be any of the 20 amino acids. Sugars
typically added in N-linked glycosylation include fucose,
10   galactosamine, glucosamine, galactose, glucose, and
mannose, as well as those listed above, with
galactosamine being the usual linking sugar.

             Therefore, potential O-linked glycosylation
sites of the PDGF A-chain can be found at positions Ser-
15   1, Thr-12, Thr-14, Ser-22, Thr-27, Ser-28, Thr-44, Thr-
49, Ser-50, Ser-51, Ser-57, Ser-63, Thr-95, Thr-96, Ser-
97, Thr-107 of Figure 1 and, additionally at Ser-113 and
Thr-125 of Figure 2. Possible N-glycosylation sites for
the A-chain occur at Asn-48 of Figures 1 and 2. The PDGF
20   B-chain contains a number of putative O-glycosylation
sites found at amino acid residues Ser-1, Ser-4, Thr-6,
Thr-18, Thr-20, Ser-26, Thr-33, Ser-50, Thr-63, Thr-88,
Thr-90, Thr-101, and Thr-109, as depicted in Figure 3.
However, there appear to be no N-glycosylation sites in
25   the B peptide. Thus, sugars can be linked to any of
these positions which are sterically accessible and in
such a way so that the biological activity of the
molecule is enhanced.

             Furthermore, the carbohydrate moiety added to
30   the PDGF molecule can consist of a single sugar or an
oligosaccharide chain composed of either the same
monomeric unit or a variety of sugars.

             Oligosaccharides for use with the instant
invention can be synthesized using techniques known in
35   the art. See, e.g., Hubbard et al., Ann Rev Biochem

(1981) 50:555-583 and Solid-Phase Synthesis (E.C. Blossey
and D.C. Neckers eds., 1975), the disclosures of which
are incorporated herein by reference. Specifically, for
solid-phase oligosaccharide synthesis, an activated

5   monomer unit, protected by a temporary blocking group
such a p-nitrobenzoate at one hydroxyl, and a persistent
blocking group such as a benzyl at the remaining
hydroxyls, is coupled to a suitably functionalized
allylic alcohol resin. The temporary blocking group is

10 removed under mild conditions which leave the persistent
blocking group attached. A simple alcoholysis reaction
is then performed to attach a new monomer unit to the
reactive end of the unit previously attached to the
resin. Further steps include the sequential deblocking

15 and coupling until an oligomer of the desired length is
obtained. The oligomer is then cleaved from the support
by oxidation and persistent blocking groups removed from
the soluble derivative.

       The synthesized oligosaccharide or monomeric

20 sugar can be added to the PDGF polypeptide by the
addition of the sugar along with appropriate enzymes,
according to methods well known in the art. See, e.g.,
Ann Rev Biochem (1987) 56:915-944 and FEBS 0620 (July
1983) 158(2):335-338. Any sugar or combination of

25 sugars, shown to increase PDGF activity as determined
herein, in an amount equivalent to the increase seen when
at least 10 moles of carbohydrate are present per mole of
PDGF dimer, will find use with the instant invention.
Methods for quantitating as well as well as identifying

30 the sugar moieties of glycoproteins are known in the art.
Such a determination can be made using a Dio LC
GlycoProtein Instrument (Dionex, Sunnyvale, CA.), as well
as by chemical determination using periodate, classical
paper chromatography on silica plates, and incorporation

35 of radioactive sugars into the polypeptide chain.

4.   Purification of Hyperglycosylated PDGF

           As explained above, isolated, recombinantly
produced PDGF has been found to exist in a heterogenous
5   state with respect to glycosylation. The heterogenous
mixture can be further purified by separating the two
PDGF forms, using agents known to react with
carbohydrate-containing molecules. For example, the
heterogenous expression product can be subjected to
10   affinity chromatography or affinity electrophoresis,
using ligands, such as lectins, useful for separating
glycoproteins. Particularly useful is a column packed
with a lectin-agarose resin, such as concanavalin A
(ConA) sepharose. The more highly glycosylated PDGF will
15   bind the column and can be competed off using an
appropriate sugar. The sugar used will depend on the
carbohydrate moieties present on the PDGF. For example,
as explained in more detail below, the hyperglycosylated
yeast expression product was found to contain mannose
20   moieties and therefore, a mannose sugar can be added to
the elution buffer. The hyperglycosylated PDGF can also
be recovered from the column by using an enzyme that will
cleave the linkage formed between the sugar moieties of
PDGF and the lectin. Enzymes useful with particular
25   sugar moieties are well known in the art.

5.   Synthetic Production of Glycosylated PDGF

           PDGF dimers can be produced synthetically,
based on the amino acid sequences shown in Figures 1, 2
30   and 3, using protein synthesis techniques well known in
the art, such as solid or solution phase peptide
synthesis. Summaries of common synthetic techniques can
be found in Stewart, J.M., and Young, J.D., Solid Phase
Peptide Synthesis, 2nd ed. (Pierce Chemical Co. 1984) and
35   Barany, G., and Merrifield, R.B., The Peptides: Analysis,

Synthesis, Biology, Vols. 1 and 2 (E. Gross and J.
Meienhofer eds., Academic Press 1980), and Bodansky, M.,
Principles of Peptide Synthesis, (Springer-Verlag 1984),
the disclosures of which are incorporated herein by
reference in their entirety.

In general, these methods consist of the
sequential addition of individual amino acids to a
growing chain. The amino or carboxyl group of the first
amino acid is typically protected or derivatized and this
group is either attached to an inert solid support or
used directly in solution. The next amino acid in the
sequence having the complementary (amino or carboxyl)
group suitably protected, is then added under conditions
favoring amide linkage formation. The protecting group
is then removed and the next protected amino acid residue
added. The cycle is repeated until the desired protein
has been generated.

The synthetically produced PDGF molecule will
have a primary structure substantially homologous and
functionally equivalent to those amino acid sequences
depicted in Figures 1, 2, or 3. Once synthesized, the
PDGF molecule can be glycosylated by the addition of
oligosaccharide units to appropriate glycosylation sites
in a reaction as described above. Alternatively, Arg,
Ser or Thr amino acid residues, already derivatized with
the appropriate sugar, can be used directly in the
synthesis of the PDGF molecule.

6. _Use and Administration of Hyperglycosylated PDGF_

Hyperglycosylated PDGF prepared according to
the invention is generally applied topically to wounds
such as cutaneous, dermal, mucosal, or epithelial wounds
in vertebrates, particularly mammals including man,
domestic and farm animals, sports animals and pets. It
may be used to treat any type of full or partial

-22-

thickness wounds (burns), radiation wounds, and ulcers
such as decubiti and cutaneous ulcers caused by vascular,
hematologic and metabolic diseases, infections, or
neoplasms.

5   The PDGF may be formulated using available
excipients and carriers in the form of a lotion, spray,
gel, ointment or as a controlled- or sustained-release
dosage form. Additional ingredients such as other growth
factors (FGF, CTAP-III, EGF, IGF-1, IGF-2, TGF-β, TGF-
10   α), buffers, local anesthetics, antibiotics, gelling
agents, and the like may be included in the formulation.
        For topical administration, which is the most
appropriate with regard to cutaneous lesions, standard
topical formulations are employed using, for example,
15   0.01-10% concentrations of PDGF. The concentration of
formulation depends,of course, on the severity of the
wound and nature of the subject. In some treatment
regimens, the dose is lowered with time to lessen
likelihood of scarring.
20   Controlled- or sustained-release formulations
of hyperglycosylated PDGF are made by incorporating the
PDGF in carriers or vehicles such as liposomes,
nonresorbable impermeable polymers such as ethylene-
vinyl acetate copolymers and Hytrel® copolymers,
25   swellable polymers such as hydrogels, or resorbable
polymers such as collagen and certain polyacids or
polyesters such as those used to make resorbable sutures
to provide for sustained release of the PDGF to the wound
site over an extended time period, typically from one day
30   to one week. Such incorporation may be particularly
desirable when the PDGF is incorporated into a wound
dressing. The mechanism of PDGF release from the
formulations may be diffusion, osmosis, leaching,
dissolution, erosion, or combinations thereof. In
35   diffusional sustained-release formulations, the PDGF

dissolves in and diffuses through the carrier or vehicle
on which it is encapsulated/dispersed. In leaching or
dissolution formulations, the PDGF is leached from the
carrier by body fluids. The concentration of polypeptide
5   in the sustained-release formulation will normally be at
least 1 $\mu g/ml$, usually between 10 $\mu g/ml$ and 10 mg/ml. In
some instances it may be desirable to continually
maintain the treatment composition at the affected area
or wound site during the healing period. This may be
10   achieved via a multiplicity of intermittent applications
of the treatment composition, or by administering the
PDGF via a sustained-release dosage form such as those
described above. In this regard, the term "continually"
denotes true continuous administration such as is
15   achieved by such sustained-release dosage forms or that
achieved by such repeated applications that provide a
pharmacokinetic pattern that mimics that achieved by true
continuous administration.

         The above disclosure generally describes the
20   present invention. A more complete understanding can be
obtained by reference to the following specific examples,
which are offered for illustrative purposes only, and are
not intended to limit the scope of the present invention
in any way.

25

Deposits of Strains Useful in Practicing the Invention
         A deposit of biologically pure cultures of the
following strains were made with the American Type
Culture Collection, 12301 Parklawn Drive, Rockville,
30   Maryland. The accession number indicated was assigned
after successful viability testing, and the requisite
fees were paid. Access to said cultures will be
available during pendency of the patent application to
one determined by the Commissioner to be entitled thereto
35   under 37 CFR 1.14 and 35 USC 122. All restriction on

availability of said cultures to the public will be
irrevocably removed upon the granting of a patent based
upon the application. Moreover, the designated deposits
will be maintained for a period of thirty (30) years from
5   the date of deposit, or for five (5) years after the last
request for the deposit; or for the enforceable life of
the U.S. patent, whichever is longer. Should a culture
become nonviable or be inadvertently destroyed, or, in
the case of plasmid-containing strains, loose its
10   plasmid, it will be replaced with a viable culture(s) of
the same taxonomic description.

Strain	Deposit Date	ATCC No.
MB2-1 (pYAGL7PB)	April 19, 1990	20911
pSV7D-PDGF A102-B1	April 22, 1987	40320
pSV7D-PDGF A103-B1	April 22, 1987	40321

C.   Experimental
The following examples further describe the
20   isolation of DNA encoding PDGF A-chain and B-chain
polypeptides, the expression of that DNA in various hosts
to produce biologically active PDGF, and the isolation of
hyperglycosylated PDGF.
In the following, "digestion" refers to the
25   enzymatic cleavage of DNA by restriction endonucleases.
Restriction endonucleases, commonly referred to as
restriction enzymes, are well characterized and commer-
cially available and were used in accordance with the
manufacturer's specifications. Digestion with
30   restriction enzymes is frequently followed by treatment
with alkaline phosphatase according to the manufacturer's
specifications to remove the terminal 5' phosphates, thus
preventing self-ligation of a vector having two
compatible ends.
35

"Fill in" refers to the enzymatic process of
creating blunt ends by repairing overhanging ends
generated by certain restriction enzymes. The repair is
a function of DNA polymerase I large fragment (Klenow)
5   and deoxynucleotide triphosphates and is used according
to manufacturer's specifications.

Gel isolation of a DNA restriction fragment
refers to the recovery of a specific fragment, electro-
phoretically separated on either an agarose gel or a
10   polyacrylamide gel (depending on size of fragment), by
either electroelution or melting and extraction of the
gel slice.

All DNA manipulations are done according to
standard procedures. See Maniatis et al., <u>Molecular</u>
15   <u>Cloning</u>, Cold Spring Harbor Laboratories, 1982. All
enzymes used are obtained from commercial sources and
used according to the manufacturer's specifications.

1.   <u>Isolation and Characterization of PDGF A- and</u>
20   <u>B-Chain cDNA</u>

A lambda-gt10 cDNA library was constructed from
poly (A)$^{+}$ RNA from the human clonal glioma cell line
U-343 (MGaC12:6 using the LiCl/urea method modified as
described by Betsholtz, C., et al., <u>Cell</u> (1984) <u>39</u>:447-
25   457. Oligo(dT)-primed synthesis of ds cDNA was performed
according to Gubler, U., and Hoffman, B.J., <u>Gene</u> (1983)
<u>25</u>:263-299. The resulting cDNA was treated with T4 DNA
polymerase and subcloned into <u>Eco</u>RI-cleaved lambda-gt10
using <u>Eco</u>RI linkers. The recombinant phage were plated
30   in <u>E. coli</u> C600hfl.

Two oligonucleotide probes, designated PDGF A-1
and PDGF A-2, were synthesized based on the known partial
amino acid sequence of PDGF A-chain. Both were made
using solid-phase phosphoramidite methodology. The
35   double-stranded probe PDGF A-1 was synthesized as two

-26-

overlapping 50-bp oligonucleotides and radiolabeled using
$[\alpha\text{-}^{32}P]$-deoxynucleoside triphosphates and Klenow fragment
of DNA polymerase I. PDGF A-2 was synthesized as a 37-
base template and a 12-base complementary primer and was

5   radiolabeled as PDGF A-1. The nucleotide sequences
(single strand) of the two probes are given below.

PDGF A-1 (86-mer)
              CCATCGAGGAGGCCGTGCCTGCAGTGTGC

10   AAGAACCCGCACCGTGATCTATGAAGATCCCCCGCTCC
              CAGGTCGACCCCACCTCCGCC

PDGF A-2 (37-mer)
              AAGCGCTGCACCGGCTGCTGCAACACCAGCAGCGTGA

15

              These probes were used to screen the library
$(2 \times 10^6$ clones). Duplicate nitrocellulose filter lifts
were hybridized with the probes at 42°C in 20% formamide,
5 x SSC, 50 mM sodium phosphate pH 7.0, 5 x Denhardt's,

20   0.10% SDS, 200 μg/ml sonicated salmon sperm DNA and
washed in 0.5 x SSC, 0.1% SDS at 42°C. Clones 13-1 and
D1 were selected from among those that hybridized to both
probes and sequenced by dideoxy nucleotide chain
termination after subcloning into M13 phage derivatives.

25   Partial nucleotide sequences of 13-1 and D1 are shown in
Figures 4 and 5, respectively.
              The longest open reading frame of D1 predicts a
PDGF A-chain precursor of 211 amino acids (shown in
Figure 5); the boxed portion designates the 125-amino

30   acid PDGF A-chain polypeptide.
              The deduced amino acid sequence of Figure 5
matches the reported partial sequence of the PDGF A-chain
obtained by amino acid sequencing except at amino acids
119, 141, 143, found to be Ile, Gln, and Ser;

35

respectively, instead of the previously assigned Val,
Arg, and Thr. The ATG codon at amino acid position 1
precedes a basic amino acid (Arg) followed by 18
hydrophobic residues. This is characteristic of a signal
5   peptide sequence and is consistent with the observation
that PDGF A-chain homodimers produced by human
osteosarcoma cells are secreted. Comparison with
preferred signal peptidase cleavage sites suggests that
processing may occur between amino acids Ala20 and Glu21.
10   The N-terminal sequence of platelet PDGF A-chain is found
at amino acid 87, indicating that a propeptide of 66
amino acids (44% charged residue) is cleaved from the
precursor to generate a 125-amino acid A-chain protein.
This cleavage occurs after a run of four basic amino
15   acids, Arg-Arg-Lys-Arg. Additional proteolytic
processing may occur in the C-terminal region.
     The corresponding open reading frame of 13-1
(Figure 4) predicts a PDGF A-chain precursor of 196 amino
acids identical in sequence to the precursor of D1 but
20   lacking 15 C-terminal residues. Again, the mature
polypeptide is boxed in Figure 4.
     cDNA for PDGF B-chain was isolated from the
same cDNA library for use in the following experiments in
which D1, 13-1, and B-chain cDNA were cloned in an
25   analogous manner, except where indicated.

     2. Yeast Expression
          Due to the ability of yeast to secrete and
process proteins, the genes for the mature PDGF A-chains
30   and B-chain were fused with the sequence of the α-factor
leader, a yeast secretory signal sequence which would
allow for secretion of PDGF. Yeast transformed with
these plasmids would be expected to synthesize a protein
containing an NH_2-terminal α-factor leader and COOH-
35   terminal PDGF chain separated by Lys-Arg. Since this

molecule is targeted for secretion, cleavage after the processing site Lys-Arg by the yeast should result in secretion of the mature growth factor. Lys-Arg is the processing site used by the natural prepro-PDGF, as well

5   as the prepro-α-factor.

2.1.   Regulatable Secretion in Yeast

PDGF B-chain protein, and the two forms of the A-chain protein, D1 and 13-1, were produced and secreted

10 by yeast strain Saccharomyces cerevisiae AB110 (Mata, ura 3-52, leu 2-04, or both leu 2-3 and leu 2-112, pep 4-3, his 4,580, cir°) transformed with yeast expression plasmids pYpA6 and pYpA134, respectively. Additionally, PDGF B-chain protein was produced in S. cerevisiae MB2-1

15 (Mata, uradelta3, leu 2-3, leu 2-112, his 3-11, his 3-15, pep 4delta, cir°) transformed with yeast expression plasmid pYAGL7PB. The plasmids contain the sequence coding for their respective mature PDGF protein along with pBR322 sequences including the origin of replication

20 and the ampicillin resistance gene, as well as yeast sequences including the 2-micron and selectable markers leu and ura genes. Expression of the mature PDGF genes is inducible and under the control of the regulatable promoter ADH2-glyceraldehyde-3-phosphate-dehydrogenase

25 (GAPDH) and α-factor terminator (see section 2.1.4). The individual plasmids are described in more detail below.

2.1.1.   Construction of pYAGL7PB: A Yeast Expression
         Vector for Hyperglycosylated PDGF B-Chain

30 Plasmid pYAGL7PB is a yeast expression vector which contains an "expression cassette" for PDGF B-chain cloned into the BamHI site of vector pAB24 (described more fully below). Vector pAB24 contains PBR322 and yeast 2 micron sequences including the E. coli amp^R and

35 yeast leu2 and ura3 genes.

The "expression cassette" consists of the
following sequenc s fused together in this order (5' to
3'): ADH2 regulatory sequences, GAP promoter, truncated
α-factor leader (EPA no. 324274), PDGF B synthetic gene,
and α-factor terminator. The map of plasmid pYAGL7PB is
shown in Figure 6. The PDGF B gene cloned into the
expression cassette was chemically synthesized employing
yeast preferred codons using the phosphoramidite
procedure as described by Urdea, et al., (Proc Natl Acad
Sci USA (1983) 80:7461) and according to the Dayhoff
protein data bank amino acid sequences for the B-chain of
PDGF. The PDGF B gene comprises a 327 bp DNA fragment
coding for 109 amino acids. The sequence is shown in
Figure 3.

2.1.2. Construction of pYpA6 and pYpA134: Yeast
        Expression Vectors for PDGF A-Chain
        In vitro mutagenesis was used to generate XbaI
and SalI sites at the end of the two different mature
PDGF A-chain genes in order to clone the genes into pAG
(see section 2.1.4). In vitro mutagenesis was performed
according to the procedure of Zoller and Smith, DNA
(1984) 3(6):479-488. A SacI-HindIII fragment, containing
the entire coding region for the mature polypeptide, was
cloned from each of the PDGF A-chain genes into M13mp19
in order to generate single-stranded template. The
following synthetic oligonucleotides were used as
mutagenic primers.

-30-

Mutation	Sequence of Primer
1. XbaI site at 5' end of 13-1 and D1	CTGCCCATTCTAGATAAGAGAAGCATC
2. SalI site at 3' end of clone D1	CCCACCTAAAGTCGACTTCCAGATGTGAGG
3. SalI site at 3' end of clone 13-1	GATGACCCGTCGACCAATCCTGGGA

5

Mutagenesis of clone D1 was carried out using
10   primers 1 and 2; primers 1 and 3 were used for clone
13-1. An approximately 400-bp XbaI-SalI (partial)
fragment from clone D1 and an approximately 360-bp XbaI-
SalI (partial) fragment from clone 13-1 were each
isolated and cloned into pAG, which was digested with the
15   XbaI and SalI and gel isolated, to give pAG-AD1 and pAG-
A13.1, respectively. The expression cassettes containing
the two forms of the mature PDGF A-chain gene were cut
out as BamHI fragments and each cloned into pAB24,
previously digested with BamHI and treated with alkaline
20   phosphatase, to give plasmids pYpA6 and pYpA134, depicted
in Figures 7 and 8, respectively.

2.1.3.   Yeast Transformation and Expression
Yeast expression plasmids pYpA6 and pYpA134
25   were transformed into yeast strain S. cerevisiae AB110
(Mata, ura 3-52, leu 2-04, or both leu 2-3 and leu 2-
112, pep 4-3, his 4-580, cir°), as described by Hinnen et
al. (Proc Natl Acad Sci USA (1978) 75:1929-1933). Yeast
expression plasmid pYAGL7PB was transformed into yeast
30   strain S. cerevisiae MB2-1 (Mata, Ura 3delta, leu 2-3,
leu 2-112, his 3-11, his-315, pep 4delta, cir°). Yeast
was plated on ura⁻, 8% glucose, sorbitol plates.
Transformants were grown in leu⁻, 8% glucose liquid
medium for 24 hr and then plated onto leu⁻, 8% glucose
35

sorbitol plates to get individual colonies. Individual
colonies were picked and grown in 3 ml of leu⁻, 8%
glucose medium for 24 hr at 30°C, and then inoculated
(1:50) into 1 liter of ura⁻, 1% glucose media and grown

5   for 75 hr at 30°C. Yeast culture medium was assayed for
PDGF activity by the human foreskin fibroblast mitogen
assay (see section 5). The yeast transformant pYpA6-NT1
secretes PDGF A-chain (D1 form) into the medium at a
level of 750 ng/ml and transformant pYpA134-NT1 secretes

10   PDGF A-chain (13-1 form) into the medium at a level of
325 ng/ml.

        For large scale production of hyperglycosylated
PDGF B-B homodimer, the procedure was as follows.

15   2.1.3.1. <u>Preparation of Master/Working Seed Stock</u>
        <u>S. cerevisiae</u> MB2-1 (pYAGL7PB) was first
streaked onto ura- selective plates and then single
transformants or a frozen glycerol stock was individually
inoculated into leu- selective medium. After 48 hours of

20   growth, at 30 degrees C, a portion of this culture was
frozen in a final volume of about 19% glycerol and frozen
in a dry ice/ ethanol bath and stored at -70°C or colder.
The other portion, used for expression testing, was grown
for about 72 hours in a medium similar to ura- selective

25   medium-2 (see below), with the exception that the glucose
level in the medium was 1% instead 8%. This was agitated
for 72 hours, at 30 degrees C. The cultures were
harvested, and cell free supernatants were prepared and
assayed for PDGF B expression levels. Aliquots from the

30   culture exhibiting the highest expression level were
selected as the seed stock.

35

2.1.3.2. Inoculum 1

A seed stock aliquot, prepared in section
2.1.3.1., was thawed and one aliquot used t inoculate
500 ml of ura- selective medium-1 in a shake flask. This
5   culture was incubated for 1 to 2 days at a controlled
temperature with agitation. Inoculum 1 may consist of
one to three 500 ml cultures. The inoculum was used
immediately to inoculate complete media in 16 L seed
fermentors. Alternatively, the inoculum was stored at
10   2-8°C for up to seven days, prior to use.

2.1.3.3. Inoculum 2

Each 500 ml culture of inoculum 1 was used to
inoculate ura- selective medium-2 in a 16 L seed
15   fermentor. The inoculum 2 was grown for about 18 hours,
at 30 degrees C, at an airflow rate of 10 LPM and an
agitation rate of 400 RPM, back pressure was one to three
psi. This was used to inoculate production medium in a
large scale fermentor. Inoculation ratios varied
20   depending on scale of production fermentor used (sizes
varied from 3,400 L to 10,000 L). One to three 16 L seed
fermentors were typically used to set a production
fermentor. The complete media contained salts, vitamins,
trace elements, casamino acids and glucose.
25

2.1.3.4. Fermentor Culture

The inoculum 2 was used to inoculate complete
production medium in a large scale production fermentor
of 3,400 L, 4,000 L, and/or 10,000 L capacity. This
30   culture was incubated with controlled temperature,
aeration, and agitation for about 72 hours. Harvest time
was determined by fermentation time alone.

35

2.1.3.5. <u>Media Used in Large Scale Pr duction</u>
          The comp nents of the various media used above
were as f llows.

5   <u>Ura- Selective Medium</u>

            Ura-/Sorbitol media   485   ml
            20% Casamino Acids   12.5   ml
            1% Tryptophan   2.5   ml
            1% Adenine   2.5   ml
10   50% Glucose   80   ml

                   <u>Ura-/Sorbitol Medium</u>

        Agar   80 g
        Yeast Nitrogen Base w/o Amino Acids   26.8 g
15   Sorbitol   728 g
        Milli-Q Water   3.6 L

                   <u>Leu- Selective Medium</u>

        10X Basal Salts w/o Amino Acids   100 ml
20   50% Glucose   160 ml
        Leu- Supplements   100 ml
        5% Thronine   4 ml
        Pantothenate/Inositol Solution   2.5 ml
        q.s. with Milli-Q Water to 1L

25   <u>Leu- Supplements</u>

            Adenine   4 g
            Uridine   3 g
            Tryptophan   2 g
            Histidine   2 g
30   Arginine   2 g
            Methionine   2 g
            Tyrosine   3 g
            Lysine   3 g
            Phenylalanine   5 g
35

10X Basal Salts

Yeast Nitrogen Base w/o Amino Acids	267.2 g
Succinic Acid	400 g
Sodium Hydroxide	240 g
q.s. with Milli-Q water to 4L	

Pantothenate/Inositol Solution

D-Pantothenic Acid Hemicalcium Salt (1.2%)	12.0 g
Myoinositol (1.2%)	12.0 g
q.s. with Milli-Q water to 4 L	

Ura- Selective Medium-1

Basal Salts (10X) w/o Amino Acids	100 ml
50% Glucose	160 ml
20% Casamino Acids	25 ml
(Sheffield Co. or Marcor)	
1% Adenine	5 ml
1% Tryptophan	5 ml
Pantothenate/Inositol Solution	2.5 ml
q.s.t o 1 L using MILLI-Q water and filter	
through 0.2 µ m filter units	

Ura- Selective Medium-2

Yeast Nitrogen Base w/o Amino Acids	100 g
Casamino Acids	200 g
Ca D-Pantothenate	500 mg
Myo-Inositol	500 mg
Glucose, 50%	1600 ml
Antifoam (SAG-471)	1.0 ml
Milli-Q water	9 L
Inoculum 1	480 ml
PDGF B	

pH to 5.6-5.8 using 75% Phosphoric Acid or 50% NaOH.

Complete Production Medium

Casamino Acids		70 kg
Ammonium Sulfate		17.5 kg
$(NH_4)_2SO_4$		
5	Monopotassium Phosphate	3.5 kg
KH_2PO_4		
Magnesium Sulfate (Epsom salts)		1.75 kg
$MgSO_4 7H_2O$		
Sodium Chloride, NaCl		350 g
10	Calcium Chloride, Anhydrous, $CaCl_2$	350 g
3400 L trace elements powder (T-24)		1 container
Antifoam (SAG 471)		28 fl. oz.
Dextrose (Cerelose 2001) (batch)		123 kg (batch)
3400 L vitamin powder (T-24)		1 container
15	P-10 water (Manteca) (batch)	815 gallons
Inoculum 2		22 L
pH to 6.0.		

3400 L Trace Elements

20	Boric Acid	5.0 g
Cupric Sulfate		0.4 g
Potassium Iodide		1.0 g
Ferric Chloride		2.0 g
Manganous Sulfate Monohydrate		3.9 g
25	Sodium Molybdate	2.0 g
Zinc Sulfate		3.9 g

30

35

<u>3400 L Vitamin Powder</u>

Thiamine	9.5 g
Pyridoxine	9.5 g
Biotin	0.6 g
p-aminobenzoic acid	6.3 g
Riboflavin	6.3 g
Folic Acid	0.6 g
Niacin	9.5 g
Myo-Inositol	115 g
Ca Pantothenate	115 g

2.1.4.   <u>Plasmids Used for the Preparation of Yeast
          Expression Vectors</u>

          pAG- is a general yeast expression cassette
vector derived from pAG-TNF where pAG-TNF is only used
for convenience of cloning with the pAG- construct being
relevant to this application.

          The expression cassette vector, pAG-TNF,
contains the regulatable ADH2-GAPDH promoter, the α-
factor leader, the synthetic TNF gene, and the α-factor
terminator cloned in pBRdeltaRI-Sal. The ADH2-GAPDH
promoter was isolated as a 1.0-Kbp <u>Bam</u>HI-<u>Nco</u>I fragment
from pAGdeltaXbaGAP1. The 5' end of the α-factor leader
was supplied as a synthetic adaptor for the following
sequence and having <u>Nco</u>I- and <u>Pst</u>I-compatible overhangs:

CATGAGATTCCTTCAATTTTTACTGCA

TCTAAGGAAGTTAAAAATG

The 3' end of the α-factor leader, the synthetic TNF gene
and the α-factor terminator was isolated as a <u>Pst</u>I-<u>Bam</u>HI
fragment from pHG100-TNF. The three fragments were
ligated together and cloned into pBRdeltaRI-Sal which had
been previously digested with <u>Bam</u>HI and treated with
alkaline phosphatase.

pBRdeltaRI-Sal was constructed by digesting
pBR322 with EcoRI and SalI, filling in the overhangs with
Klenow fragment, and ligating on BamHI linkers. The
vector and linkers were digested with BamHI and the
5   BamHI-BamHI 3.8 kb vector was gel isolated and
recircularized by self-ligation. The resulting plasmid
was designated pBRdeltaRI-Sal.

The plasmid pAGdeltaXbaGAP1 contains the ADH2-
GAPDH hybrid promoter and the GAPDH terminator cloned
10   into pBRdeltaRI-Sal. The ADH2-GAPDH promoter (the only
sequence pertinent to this application) was isolated as a
1.1-kb BamHI-NcoI fragment from pJS103 (described below).
In addition, an approximately 90-bp XbaI-XbaI deletion
was introduced into the 5' end of the promoter fragment
15   by cutting the plasmid with XbaI, filling in the overhang
ends with Klenow fragment, and dNTPs and recircularizing
the plasmid, thus giving pAGdeltaXbaGAP1.

pHG100-TNF contains the α-factor promoter,
leader, and terminator with the synthetic gene coding for
20   TNF inserted in frame at the 3' end of the leader. The
1.0 kb Pst-BamHI fragment isolated from this plasmid
contains 240 bp of the 3' end of the α-factor leader, the
494-bp synthetic TNF gene (as an XbaI-SalI fragment) and
the 272-bp α-factor terminator. The α-factor sequences
25   which are the only sequences relevant to this application
are derived from pAB114. pAB114 is described in EPO
0 116 201, pages 14-18, and Brake, A.J., et al., Proc
Natl Acad Sci USA (1984), 81:4642-4646. The only
difference is that a silent mutation was introduced by
30   M13 mutagenesis to create an XbaI site at the 3' end of
the leader to facilitate cloning of heterologous genes.

The comparison of the 3' end of the α-factor
leader from the wild type (pAB114) versus the altered
α-factor (pHG100) illustrated below shows that a silent
35   mutation was incorporated to code for an XbaI site just

5' to the processing site (Lys-Arg). This allows for insertion of heterologous genes without the "spacer" codons (must provide the Lys-Arg processing site and maintain reading frame).

5

10

15

20

25

30

35

5

10

15

20

25

30

35

α-factor leader   processing site   spacer   α-factor gene

IleAlaAlaLysGluGluGlyValSerLeuAspLysArgGluAlaGluAlaTrpHisLeuGlnLeuGly

pAB114   ATTGCTGCTAAAGAAGAAGGGGTATCTTTGGATAAAAGAGAGGCTGAAGCTTGGCATTGGTTGCAACTGGGGG

                       <u>XbaI</u>   <u>HindIII</u>

pHG100   ATTGCTGCTAAAGAAGAAGGGGTATCTCTAGATAAAAGAGCTCCAACCTCTTCCTCTACCAAGAAGACCCAG

IleAlaAlaLysGluGluGlyValSerLeuAspLysArgAlaProThrSerSerThrLyslysThrGln

α-factor leader   processing site   gene for IL-2

pJS103

Plasmid pJS103 contains the inducible hybrid
ADH2-GAPDH promoter. The hybrid promoter is made up of
the transcriptional and translational initiation region
5   from the GAPDH promoter and is under the regulatory con-
trol of the ADH2 transcriptional regulatory region. The
ADH2 transcriptional regulatory region is derepressed in
the absence of a readily available source such as glucose
(without exogenous inducer). By allowing for glucose
10   exhaustion after the yeast culture is grown to high
density, the transcriptional control region will be
derepressed and expression of the desired peptide will
occur.

Plasmid pJS103 was constructed as follows. The
15   ADH2 portion of the promoter was constructed by cutting a
plasmid containing the wild-type ADH2 gene from plasmid
pADR2 (Beier et al., Nature (1982) 300:724-728) with
restriction enzyme EcoRV, which acts at position +66
relative to the ATG start codon, as well as in two other
20   sites in pADR2, outside of the ADH2 region. The
resulting mixture of a vector fragment and two smaller
fragments was resected with Bal31 exonuclease to remove
about 300 bp. Synthetic XhoI linkers were ligated onto
the Bal31-treated DNA. The resulting DNA linker vector
25   fragment (about 5 kb) was separated from the linkers by
column chromatography, cut with restriction enzyme XhoI,
religated, and used to transform E. coli to ampicillin
resistance. The positions of the XhoI linker were
determined by DNA sequencing. One plasmid which
30   contained an XhoI linker within the 5' nontranscribed
region of the ADH2 gene (position -232 from ATG) was cut
with the restriction enzyme XhoI, treated with nuclease
S1, and subsequently treated with the restriction enzyme
EcoRI to create a linear vector molecule having one blunt
35   end at the site of the XhoI linker and an EcoRI end. The

GAP portion of the promoter was constructed by cutting plasmid pPGAP1 with the enzymes BamHI and EcoRI, followed by the isolation of the 0.4 Kbp DNA fragment. This purified fragment was then completely digested with the enzyme AluI and an approximately 200 bp fragment was isolated.

This GAP promoter fragment was ligated to the ADH2 fragment present on the linear vector described above to give plasmid pJS103.

pPGAP1

pPGAP1 is a yeast expression cassette vector which has a polyrestriction site linker between the GAPDH terminator and a truncated GAPDH promoter region. The polyrestriction site contains the recognition sites for NcoI, EcoI, and SalI, and the cassette is excisable as a BamHI fragment. The preparation of pPGAP1 is described in EPO 164556 and Travis, J., et al., J Biol Chem (1985) 260(7):4384-4389. In both references pPGAP1 is referred to pPGAP.

pAB24

Plasmid pAB24 (Fig. 9) is a yeast shuttle vector which contains the complete 2μ sequence (Broach, Molecular Biology of the Yeast Saccharomyces (1981) Vol. 1, p. 445, Cold Spring Harbor Press) and pBR322 sequences. It also contains the yeast Ura 3 gene derived from plasmid YEp24 (Bostein et al., Gene (1979) 8:17) and the yeast LEU^{2d} gene derived from plasmid pCl/1. EPO Publ. No. 116,201. Plasmid pAB24 was constructed by digesting YEp24 with EcoRI and religating the vector to remove the partial 2μ sequences. The resulting plasmid, YEp24deltaRI, was linearized by digestion with ClaI and ligated with the complete 2μ plasmid which had been linearized with ClaI. The resulting plasmid, pCBou, was

then digested with <u>Xba</u>I and the 8605 bp vector fragment
was gel isolated. This is lated <u>Xba</u>I fragment containing
the LEU^{2}d gene isolated from pCl/1; the orientation of
the LEU^{2}d gene is in the same direction as the URA3 gene.

5   Insertion of the expression cassette was in the unique
<u>Bam</u>HI site of the pBR322 sequences, this interrupting the
gene for bacterial resistance to tetracycline.

3. <u>Mammalian Cell Expression</u>

10   In order to establish a permanent cell line
producing PDGF, the entire cDNA was cloned into a
mammalian cell expression vector which contains a
transcriptional regulatory element, a polyadenylation
site, and a transcriptional terminator signal. The

15 resulting plasmid along with a selectable marker was
introduced into Chinese hamster ovary cells (CHO).

3.1 <u>Constructions of Mammalian Cell Expression Vectors</u>
<u>pSV7d-PDGF-A102, pSV7d-PDGF-A103, and pSV7d-PDGF-B1</u>

20   Three separate mammalian cell expression
vectors were constructed by isolating <u>Eco</u>RI fragments
from each of the three cDNA clones and ligating them into
pSV7d (see §3.4 below) previously digested with <u>Eco</u>RI and
treated with alkaline phosphatase. The resulting clones

25 pSV7d-PDGF-A103 (DdDDD1), pSV7d-PDGF-A102 (13-1), and
pSV7d-PDGF-B1 (B chain) were isolated and characterized
by restriction digests. These plasmids were used to
produce the chimeric plasmids pSV7d-PDGF-A102-B1 and
pSV7d-PDGF-A103-b1 for coexpression of B chain and A

30 chain. Large-scale plasmid preparations were carried out
for all of the constructions described. The DNA was used
to transfect CHO cells.

35

3.2   CHO Cell Transfections
          Transfections were performed as follows:
          CHO dhfr⁻ cells (Urlaub and Chasin, Proc Natl
Acad Sci (USA) (1980) 77:4216) were plated at a density
of 5 x 10⁵ to 10⁶ cells per 10-cm dish prior to
transfection in nutrient medium (F12 supplemented with
1.18 mg/ml Na_2CO_3, 292 μg/ml glutamine, 110 μg/ml sodium
pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin, 200
μg/ml proline, and 10% FCS). The CHO cells were
transfected with each of the pSV7d-PDGF expression
plasmids that were mixed with plasmid pAD-dhfr, which
bears a selectable marker (a dhfr gene driven by the
adenovirus major late promoter, see below), using a
modification of the procedure described by Graham and van
der Eb, Virology (1973) 52:456-467. The samples,
containing a total of 10 μg of plasmid DNA, were added to
the dishes and allowed to settle onto the cells in a
carbon dioxide incubator (37°C). Six hours later, the
supernatants were aspirated, the cells rinsed gently with
Ca- and Mg-free phosphate-buffered saline (PBS-CMF), and
the dishes exposed to 15% glycerol as an adjuvant for
3.5-4 min. The cells were then rinsed gently and fed
with the above-described medium.
          Forty-eight hours after the addition of DNA to
the cells, the cells were split 1:20 into selective
medium (DMEM supplemented with a 1:1 mixture of fetal
calf serum and dialyzed fetal calf serum in addition to
the components described above). After growth in
selective medium or 1-2 weeks, colonies appeared and were
isolated and grown individually. Assays for PDGF were
performed on each of the clones (as described below).
          Transfections in which pSV7d-PDGF-A103 plus
pSV7d-PDGF-B1 and pSV7d-PDGF-A102 plus pSV7d-PDGF-B1 are
coprecipitated were also performed in addition to

transfections with the chimeric plasmids described in
§3.1 in order to establish cell lines that are producing
PDGF as a heterodimer of A-chain and B-chain.

5   The plasmid pAD-dhfr, bearing the mouse
dihydrofolate reductase (dhfr) gene was constructed by
fusing the major late promoter from adenovirus-2 (Ad-
MLP, map units 16-17.3) to the mouse dhfr cDNA (Subramani
et al., J Mol Cell Biol (1982) 1:584-864) at the 5' end.
DNA coding for the intron for SV40 small t antigen and
10   the SV40 early region polyadenylation site was obtained
from pSV2-neo (Southern and Berg, J Mol Appl Genet (1982)
1:327-341), and fused to the 3' end of the dhfr cDNA.
These three segments were subclones into pBR322 to obtain
the plasmid pAD-dhfr.

15   Several of the primary CHO transfected cell
lines secreted PDGF into the medium at levels of 1-2
ng/ml/24hr as determined by the mitogen assay described
in §5.

           Several primary clones from each of the
20   transfected lines were selected for amplification in
increasing amounts of methotrexate, 0.05 and 0.1, and 1.0
μM concentrations. Amplification and selection of
methotrexate-resistant colonies were performed according
to Kaufman, R.S., and Sharp, P.A., J Mol Biol (1982)
25   159:601-621.

3.3 Results of PDGF Expression
           Results of PDGF expression from CHO cells are
summarized in Table 1 below. Media from CHO cell lines
30   transfected with PDGF expression plasmids were assayed by
the PDGF mitogen assay described in §5. The results of
these experiments indicate that the PDGF A-chain
homodimer is active.

35

Table 1

CHO Cells Transfected with PDGF Expression Plasmids: Level of Active PDGF Secreted into Media

5   EXPERIMENT 1

Cell Lines	Plasmid	Level of PDGF/24 hr
Primary Cell Lines	pSV7d-PDGF-A102	1-2 ng/ml
	pSV7d-PDGF-A103	no cells survived
	pSV7d-PDGF-B1	1-2 ng/ml

10 Amplified cell lines
methotrexate
    level: 0.1 μM

	pSV7d-PDGF-A102	50-100 ng/ml
	pSV7d-PDGF-B1	100-150 ng/ml

Methotrexate
    level: 1 μM

	pSV7d-PDGF-A102	50-100 ng/ml
	pSV7d-PDGF-B1	100-150 ng/ml

15

EXPERIMENT 2

Primary Cell Lines	pSV7d-PDGF-A102	10-20 ng/ml
	pSV7d-PDGF-A103	1-2 ng/ml
	pSV7d-PDGF-B1	10-20 ng/ml
	pSV7d-PDGF-A102 + pSV7d-PDGF-B1	1-2 ng/ml
	pSV7D-PDGF-A103 + pSV7d-PDGF-B1	1-2 ng/ml

20

Amplified Cell lines

25 methotrexate level:
    1 μM & 2 μM   No increase in level of PDGF
                         secreted into media.

EXPERIMENT 3

30 Primary Cell Lines	pSV7D-PDGF A102-B1	40 ng/ml
	pSV7D-PDGF A103-B1	no secretion detected

35

3.4 Construction of pSV7d

        The mammalian cell shuttle vector plasmid pSV7d
contains the SV40 origin of replication and early
promoter (315 bp, PvuII pos 272-StuI pos 5193 with an 8

5   bp deletion between nucleotides 173 and 182), a
polylinker, and the SV40 poly A addition site (217 bp,
BclI pos 2775-pos 2558). Buchman, L., et al., "The SV40
Nucleotide Sequence," pp. 7799-841, DNA TUMOR VIRUSES,
Second Edition, edited by Tooze, J. The sequence of the

10   SV40 region is shown in Figure 13. The SV40 sequences
were cloned into the pBR322 derivative pML (Lusky and
Botchan, Cell (1984) 36:391) between nucleotide 4210 and
NruI pos 973. Maniatis, T., et al., "Nucleotide Sequence
of pBR322," (1983) Molecular Cloning: A Laboratory

15   Manual. The SV40 sequences are positioned such that the
direction of transcription from the early promoter is in
the same direction as the ampicillin gene of the vector.
A map of the plasmid is shown in Figure 14.

20   4. Isolation of Hyperglycosylated PDGF Expressed in
        Yeast

        PDGF B-B homodimer produced in S. cerevisiae
MB2-1, as described above, exists in a heterogenous state
with respect to glycosylation. That is, upon further

25   separation, at least two PDGF forms can be
distinguished--one being highly glycosylated and the
other form having little or no carbohydrate moieties
attached. The more highly glycosylated fraction is
dramatically more active as determined by the mitogenic

30   assay described in §5.2, below. PDGF B-B homodimer was
purified and the hyperglycosylated fraction isolated as
follows.

35

4.1.   Purification of PDGF B-B from S. cerevisiae MB2-1

Hyperglycosylated PDGF was r covered from
S. cerevisiae MB2-1 as follows. Cells were separated
from the supernatant using a Westphalia continuous flow
5   through centrifuge. The cells were treated and discarded
and the supernatant was recovered and stored chilled to
await filtration. The supernatant was then passed
through a 100k ultrafilter. The permeate was collected
and stored chilled.

10   Ion-exchange chromatography was performed to
recover and concentrate PDGF from the filtered
supernatant. The supernatant was adsorbed to an AMF Zeta
Prep SP 3000 or 32L ion-exchange cartridge under
conditions where PDGF binds to the resin. The cartridge
15   was washed with buffer and equilibrated with 0.02M acetic
acid. The equilibrated cartridge was loaded with the
100K permeate. The cartridge containing the bound PDGF
may be stored up to 72 hours at 2-8°C after loading.

To elute the bound PDGF, the cartridge was
20   first washed with 0.05M acetic acid/0.5M sodium chloride
to remove contaminant proteins. The optical density was
monitored at 254 nm. Elution of PDGF B-B occurs with
0.05M sodium phosphate/1M sodium chloride, pH 7.5. All
major peaks as determined by monitoring OD_{254} were
25   collected and analyzed for PDGF B content by analytical
Reverse Phase HPLC (RP HPLC). All eluate peaks
containing substantial amounts of PDGF B were pooled.
The purity of the eluted fractions at this step ranged
from 40% to 80%. The pooled eluates may be stored up to
30   72 hours at 2-8°C before further processing.

The pooled SP cartridge eluate was titrated to
pH 8 with 6N hydrochloric acid or 6N sodium hydroxide as
needed. A copper chelate sepharose column was charged
with Cu^{++} by the application of 0.1 M copper sulfate.
35

The column was then washed thoroughly with 10% acetic
acid/1M sodium chloride. The prepared load was pumped
onto the column which had been equilibrated with 0.05M
sodium acetate/1M sodium chloride, pH 8. The eluate was
5   monitored at 254 nm. The loaded column underwent a
series of washes beginning with 0.05M sodium acetate/1M
sodium chloride, pH 8. The next wash consisted of 0.05M
sodium acetate/ 0.05M sodium chloride, pH 8. The third
wash titrated the column to pH 5 with 0.05M sodium
10   acetate/0.05M sodium chloride, pH 5. The elution buffer
was 0.05M sodium acetate/0.5M sodium chloride, pH 5.
Fractions of the eluate peak were collected and analyzed
for PDGF B content and purity by RP HPLC. Purity of the
pooled fractions at this step ranged from 54% to 90%.
15   The pool may be stored up to 72 hours at 2-8°C prior to
further processing.
        The pooled copper chelate eluate was
concentrated and diafiltered using a 10K ultrafilter to
eliminate excess salt and Cu^{++}. Once the volume had been
20   reduced and the PDGF B concentration was between 2 to
5 mg/ml, the retentate was diafiltered with purified
water until the conductivity level measured in the
permeate was less than 0.1 mS/cm (10 to 20 retentate
volumes).
25   The PDGF B fraction from above was then
subjected to sulfopropyl HPLC. The SP column was
equilibrated with 0.05M sodium phosphate, monobasic. The
starting material was titrated to pH 4.2 using phosphoric
acid. After loading, the column was washed with the
30   equilibration buffer. The column was then washed with
0.05M sodium phosphate, pH 7. The PDGF B was eluted with
a linear gradient of 0 to 1M sodium chloride in 0.05
sodium phosphate over 15 column volumes. Fractions were
collected and analyzed by RP HPLC. The pooled SP HPLC
35

-49-

fractions may be stored up to 48 hours at 2-8°C before further processing.

The final pr duct was concentrated and diafiltered with purified water (P30) on a 10K AG Technology hollow fiber ultrafilter. The finished bulk product had a concentration of 2 to 5 mg/ml and was free of excess salt. The ultrafiltration circuit consisted of the same components specified in the first ultrafiltration step. The retentate was again diafiltered until the permeate conductivity was less then 0.1 mS/cm. The retentate was then passed through a 0.2 micron filter. The product may be stored lyophilized.

4.2. Isolation of Hyperglycosylated PDGF B-B Homodimer

As explained above, the purified PDGF B product from above exists in a heterogenous state with respect to degree of glycosylation. Lots tested contained from about 50% highly glycosylated PDGF to about 75% highly glycosylated PDGF, as determined by ConA affinity chromatography. Table 2 shows the composition of the heterogenous mixture recovered in Example 4.1, including the percentage of highly glycosylated and less or nonglycosylated PDGF, as well as the activity of these fractions.

Table 2

<u>Composition of Purified PDGF B</u>
<u>From Example 3.1</u>

| | | | | <u>Percent Activity[1]</u> | |
Lot #	% Highly glyco. PDGF[2]	% less or nonglyco.	Mixture	Highly glyco. PDGF	Less or nonglyco.
MEC109	55	45	67	105	48.5
MEC508	50	50	94	84.1	36.5
MEC577	52	58	100	106.5	47
MEC710	54	46	88.5	127.4	35
MEF043	70	30	177	--	--
MEF593	73	27	83	--	--

[1] Activity was determined using the HFF mitogen assay
described herein. Numbers were generated based on a PDGF
B standard that was assigned 100% activity.

[2] Fractions were separated using ConA affinity
chromatography as described herein.

As can be seen, the less or nonglycosylated
fractions were consistently, significantly lower in
biological activity than the highly glycosylated
fraction, the glycosylated fraction exhibiting as much as
three times the activity as the less glycosylated
fraction. Figure 10 also shows that as percent
glycosylation increases, so does activity.

ConA affinity chromatography was used to
separate the glycosylated and less glycosylated fractions
from one another as follows. ConA-Sepharose 4B
(Pharmacia) was packed into a 15 cm x 1 cm column to a
final volume of 13 ml. The lyophilized products from

§4.1 were diluted in water to a concentration of
approximately 1 mg/ml and loaded onto the column. After
the protein was loaded, the column was washed with
equilibration buffer to eliminate any protein which did
5   not bind to the resin. The glycosylated protein was then
eluted off the column with 0.4M α-methyl-D-
mannopyranoside (sigma) in equilibration buffer.
Affinity chromatography with ConA-Sepharose yielded two
protein fractions. One did not bind to the resin, and
10   the other was eluted off the column only after treatment
with 0.4M α-methyl-D-mannopyranoside. A typical
chromatogram is shown in Figure 11.

To determine whether mannose was the
carbohydrate present, the uptake of ^{3}H-mannose by PDGF
15   B-producing cells was measured. Specifically, these
cells were grown at 30°C in 25 ml of Leu minus media
containing 8% glucose for 48 hours. Two ml of this
growth was then transferred to 100-500 ml of Ura minus
media with 1% glucose. At 43 hours, 150-840 μCi of D-
20   [2,6-^{3}H]-mannose (Amersham) was added to the media and
the growth was continued. The cells were harvested at 70
hours by centrifugation at 5000 g for 20 minutes at 4°C.
The supernatant was collected and acidified by the
addition of glacial acetic acid to a final concentration
25   of 50 mM. Seventy-five μg of nonradioactive PDGF was
then added to the supernatant as a carrier. The
supernatant was loaded onto a 7 ml S-Sepharose fast flow
(Pharmacia) column which had been equilibrated with 50 mM
acetic acid/0.12 M NaCl. Following the loading, the
30   column was washed with equilibration buffer followed by a
wash with 50 mM acetic acid/0.5 M NaCl. The PDGF was
then eluted off the column with 50 mM acetic acid/1.0 M
NaCl. Fractions collected during the second wash and the
elution were counted to determine if the in vivo
35

-52-

incorporation was successful. A control experiment was
also carried out in which the ^{3}H-mannose was added to the
supernatant after centrifugation to determine if there
was any nonspecific binding to PDGF. The results
5   indicate that no mannose is associated with PDGF unless
it is covalently bound to the molecule.

The in vivo incorporation of D-[2,6-^{3}H]-mannose
was successful. The results of this experiment, are
illustrated in Figure 12. As can be seen, mannose
10   appears to be the carbohydrate which is involved in the
glycosylation of PDGF expressed in yeast.

To confirm this, deglycosylation was carried
out by treating PDGF with α-mannosidase (Boehringer
Mannheim) in a ratio of 10:1. The reaction was carried
15   out in 50 mM Na Acetate/10 mM ZnCl$_2$ pH 4.0 at 37°C. The
reaction was allowed to take place over a period of five
days, and was stopped by boiling the solution for 3
minutes. The boiled solution was then passed through a
Bio Gel P-10 (BioRad) gel filtration column, equilibrated
20   with 50 mM acetic acid. The PDGF-containing peak was
pooled and dialyzed against dH$_2$O to insure elimination of
all free mannose residues. The final product was
subjected to analysis by SDS-PAGE, mitogenic assay, and
mannose determination by periodate (as explained below).
25   Since PDGF B has a number of potential O-linked
glycosylation sites, deglycosylation was also attempted
by ß-elimination. This was done by suspending PDGF in
0.1 N NaOH and incubating at 37°C for 48 hours.
Following incubation, the protein was passed through a
30   Bio Gel P-10 column, equilibrated with 50 mM acetic acid,
to desalt and remove any free mannose residues. The PDGF
peak was pooled, dialyzed against dH$_2$O, and tested for
biological activity as well as for the presence of
mannose by the periodate reaction. The elimination
35

reaction resulted in the removal of counts from the
protein, indicating that radioactive mannose had been
removed.

The periodate method was used to determine the
number of moles of mannose per mole of PDGF in the highly
and less glycosylated fractions from Experiment 4.1 as
well as in the deglycosylated products above. This
method, well known in the art, calls for the oxidation of
the sugar by periodate, followed by a colorimetric
determination of the residual periodate not utilized
during the oxidation process (see Avigad, G.,
Carbohydrate Research (1969) 11:119-123, the disclosure
of which is incorporated herein by reference). Analysis
by the periodate reaction showed that the fraction that
did not bind to the ConA resin was indeed less
glycosylated than the protein which did bind. On the
average, the less glycosylated fraction contains from 2-
5 moles of mannose/mole of PDGF. The more glycosylated
protein had a higher apparent molecular weight, as
determined by SDS-PAGE.

Furthermore, deglycosylation with α-mannosidase
gave both a decrease in the number of moles of
mannose/mole of PDGF as well as in the mitogenic
activity. Before treatment with α-mannosidase there were
9 moles of mannose/ mole of PDGF as determined by the
periodate method. After the deglycosylation reaction,
there were only 6.5 moles of mannose/mole of PDGF. This
figure would not be expected to go to zero since the α-
mannosidase only cleaves the external mannose residues
and not the internal ones. After the reaction, the
biological activity went down to approximately 60% of the
original activity. These results support the theory that
glycosylation plays a role in the biological activity of
PDGF. The SDS-PAGE results also show a lower molecular

weight protein following the deglycosylation reaction
with α-mannosidase.

After β-elimination, the periodate reaction
showed a decrease in moles of mannose from 9 moles to
.8-6 moles. The biological activity, however, was
destroyed under these harsh conditions.

To determine which amino acid residues are
glycosylated when PDGF B is produced in yeast, a
heterogenous mixture containing approximately 70% highly
glycosylated material was subjected to tryptic digestion
and the tryptic fragments weighed and sequenced. Several
fragments were heavier than the theoretical molecular
weight of the amino acid residues contained therein, the
increase in weight being in exact increments of the
weight of mannose. Results varied, and it was sometimes
difficult to determine which residue was indeed
glycosylated since some tryptic fragments contained more
than one glycosylation site. However, preliminary
experiments indicate that the highly glycosylated PDGF
appears to have from 0-2 mannoses on Thr-6, 0-1 on Thr-
20, 0-1 on Ser-26, 0-2 on Thr-63, 0-3 on Thr-88, 0-3 on
Thr-90, and 0-1 on Thr-101.

This determination was also carried out on a
PDGF B heterogeneous mixture having a higher percentage
of less glycosylated material than hyperglycosylated
PDGF. No sugar groups were found at Thr-20, Ser-26 or
Thr-101. Therefore, it is hypothesized that a purified
sample of the less glycosylated PDGF B would lack sugars
on these residues.

5.   Bioassay for PDGF Activity

5.1.   PDGF Produced in Mammalian Cells

          Chinese hamster ovary cells (CHO) used in the
5   process were normally grown in medium supplemented with
     10% fetal calf serum (FCS). This presented a problem in
     assaying for PDGF due to the background contributed by
     the native bovine PDGF in FCS. Thus, it was necessary to
     devise culture conditions that support production of
10   recombinant products while reducing the background.
     Using CHO cells transfected with the human ß-interferon
     gene, it was found that expression levels reached
     approximately 50% of those observed in 10% FCS with
     culture medium supplemented by 5% platelet-deficient
15   horse plasma (PDHS). This medium, when added to either
     the cell growth or mitogen assays, gave no background.
          CHO transformants were assayed by adding 10 µl
     of a 24-hr supernatant harvest in 5% PDHS (and necessary
     dilutions, usually serial twofold and threefold) to the
20   well of a 96-well plates of the assay.

5.2.   PDGF Produced in Yeast

          Samples of supernatants of yeast which had
25   expressed PDGF were appropriately diluted, depending upon
     expected activity, and then serially diluted in DMEM
     containing 1% bovine serum albumin (BSA). Aliquots
     (10 µl) of each dilution were placed in the wells of the
     assay plates.

30

5.3   Human Foreskin Fibroblast (HFF) Mitogen Assay for
      PDGF

          HFF stocks were stored frozen; freezing was at
     passage 13. Prior to use, HFF were thawed, and grown in
35   T75 flasks until confluent, which usually occurred at 5-7

-56-

days. Growth medium contained Dulbecco's Modified Eagles
Medium (DMEM), 20% fetal bovine serum (FBS), 1 mM sodium
pyruvate, 300 μg/ml L-glutamine, 100U/ml penicillin, and
100 μg/ml streptomycin. Cells were incubated at 37°C in

5   humidified 7% CO_2, 93% air atmosphere. At confluency,
cells were passaged by rinsing the monolayer with
phosphate buffered saline (PBS) lacking Ca^{++} and Mg^{++},
dissociating them in trypsin containing EDTA, and
diluting them with Growth Medium. Cells were passaged no

10   more than 8 times after thawing.

To assay for PDGF, HFFs were plated as follows.
The cells were rinsed and dissociated with trypsin as
above. The trypsinized cells were pelleted, and resus-
pended to a concentration of 1 x 10^5 cells/ml in medium

15   similar to Growth Medium, except that 5% FBS replaced 20%
FBS; 100 μl of suspension was dispensed into each well of
a 96 well microtiter plate, and the cells were incubated
5-6 days under the above described conditions.

PDGF in the sample was determined by monitoring

20   ^{3}H-thymidine incorporation into HFF DNA stimulated by
PDGF. Samples were added to the wells containing HFF
monolayers, and the assay plates incubated as above for
18 hours. The HFF cultures were then pulsed with
[Methyl-^{3}H] thymidine (10 μC/ml final concentration,

25   1 μC/well) at 37°C under the above described incubation
conditions for 8 hours. After incubation, the cells were
rinsed with PBS and fixed. Fixing was by incubation with
5% trichloracetic acid (TCA) and then 100% methanol for
15 minutes, followed by drying in air. The cells were

30   then solubilized with 0.3N NaOH, and counted in a liquid
scintillation counter.

Control samples were treated as the samples
described above, and were prepared as follows. For
positive controls used in activity tests of PDGF produced

35

in <u>S. cerevisiae</u> MB2-1, PDGF B-B homodimer from
<u>S. cerevisiae</u> MB2-1, transformed with pYAGL7PB, was
dissolved to a final concentration of 56 ng/ml in DMEM
containing 1% BSA. A standard curve was prepared; the
first point was 5.6 ng/ml, the remaining points were
2-fold serial dilutions. For positive controls in all
other tests, PDGF, purchased from PDGF, Inc., was
dissolved to a final concentration of 100 ng/ml in DMEM
containing 10 mg/ml BSA. A standard curve was prepared;
the first point was 10 ng/ml, the remaining points were
2-fold serial dilutions.

Each dilution was tested in triplicate.
Negative controls, in 1:10 dilution of 5% FCS, which
lacked both sample and control PDGF, were also run. As
can be seen in Table 2, and explained above,
hyperglycosylated PDGF exhibited far more biological
activity than its less or nonglycosylated counterpart.

Thus, hyperglycosylated PDGF and methods for
isolating the same have been disclosed. Although
preferred embodiments of the subject invention have been
described in some detail, it is understood that obvious
variations can be made without departing from the spirit
and the scope of the invention as defined by the appended
claims.

Claims

1.   A composition of hyperglycosylated PDGF.

5   2.   The composition of claim 1 wherein said
hyperglycosylated PDGF includes at least an average of 10
moles of carbohydrate per mole of dimer.

3.   The composition of claim 1 wherein said
10 hyperglycosylated PDGF includes at least an average of 17
moles of carbohydrate per mole of dimer.

4.   The composition of claim 2 wherein said
hyperglycosylated PDGF comprises PDGF B-B homodimer.
15

5.   The composition of claim 3 wherein said
hyperglycosylated PDGF comprises PDGF B-B homodimer.

6.   The composition of claim 4 wherein said
20 carbohydrate comprises mannose.

7.   The composition of claim 5 wherein said
carbohydrate comprises mannose.

25   8.   The composition of claim 1 wherein said
hyperglycosylated PDGF is produced in yeast.

9.   A PDGF B-B homodimer having at least 17
moles of mannose per mole of dimer.
30

10.   The PDGF of claim 9 wherein said PDGF is
produced in yeast.

11.   A method of producing hyperglycosylated
35 PDGF comprising:

        (a) providing a mixture containing a
hyperglycosylated PDGF fraction and a less glycosylated
PDGF fraction; and

        (b) isolating said hyperglycosylated PDGF from
said mixture.

        12.   The method of claim 11 wherein said
hyperglycosylated fraction is isolated using lectin
affinity chromatography.

        13.   The method of claim 11 wherein said
hyperglycosylated PDGF includes at least an average of 10
moles of carbohydrate per mole of dimer.

        14.   The method of claim 11 wherein said
hyperglycosylated PDGF includes at least an average of 17
moles of carbohydrate per mole of dimer.

        15.   The method of claim 13 wherein said
hyperglycosylated PDGF comprises PDGF B-B homodimer.

        16.   The method of claim 14 wherein said
hyperglycosylated PDGF comprises PDGF B-B homodimer.

        17.   The method of claim 15 wherein said
carbohydrate comprises mannose.

        18.   The method of claim 16 wherein said
carbohydrate comprises mannose.

        19.   The method of claim 11 wherein said
mixture is derived from yeast cells expressing PDGF.

20.   A method of producing PDGF B-B homodimer
having at least 17 moles of mannose per mole of dimer
substantially free f less glycosylated PDGF comprising:

(a) providing a mixture containing said PDGF B-
5   B homodimer and less glycosylated PDGF; and

(b) isolating said PDGF B-B homodimer using
lectin affinity chromatography.

21.   The method of claim 20 wherein said PDGF
10   is produced in yeast.

22.   In a method of purifying recombinant PDGF
from an expression media which comprises a
hyperglycosylated PDGF fraction and a less glycosylated
15   PDGF fraction, the improvement comprising isolating said
hyperglycosylated PDGF fraction from said expression
media.

23.   The method of claim 22 wherein said
20   hyperglycosylated PDGF is isolated using lectin affinity
chromatography.

24.   The method of claim 22 wherein said
recombinant PDGF is purified from yeast expression media.
25

30

35

1/16

Translation of PDGF A Short Form

```
AGC ATC GAG GAA GCT GTC CCC GCT GTC TGC AAG ACC AGG ACG GTC ATT
Ser Ile Glu Glu Ala Val Pro Ala Val Cys Lys Thr Arg Thr Val Ile
 1                                    10

TAC GAG ATT CCT CGG AGT CAG GTC GAC CCC ACG TCC GCC AAC TTC CTG
Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro Thr Ser Ala Asn Phe Leu
             20                                        30

ATC TGG CCC CCG TGC GTG GAG GTG AAA CGC TGC ACC GGC TGC TGC AAC
Ile Trp Pro Pro Cys Val Glu Val Lys Arg Cys Thr Gly Cys Cys Asn
                         40

ACG AGC AGT GTC AAG TGC CAG CCC TCC CGC GTC CAC CAC CGC AGC GTC
Thr Ser Ser Val Lys Cys Gln Pro Ser Arg Val His His Arg Ser Val
     50                                        60

AAG GTG GCC AAG GTG GAA TAC GTC AGG AAG AAG CCA AAA TTA AAA GAA
Lys Val Ala Lys Val Glu Tyr Val Arg Lys Lys Pro Lys Leu Lys Glu
                         70                                    80

GTC CAG GTG AGG TTA GAG GAG CAT TTG GAG TGC GCC TGC GCG ACC ACA
Val Gln Val Arg Leu Glu Glu His Leu Glu Cys Ala Cys Ala Thr Thr
                                     90

AGC CTG AAT CCG GAT TAT CGG GAA GAG GAC ACG GAT GTG AGG
Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp Thr Asp Val Arg
             100                                 110
```

Translated Mol. Weight = 12517.32

Figure 1

Translation of PDGF A Long Form

AGC ATC GAG GAA GCT GTC CCC GCT GTC TGC AAG ACC AGG ACG GTC ATT
Ser Ile Glu Glu Ala Val Pro Ala Val Cys Lys Thr Arg Thr Val Ile
1   10

TAC GAG ATT CCT CGG AGT CAG GTC GAC CCC ACG TCC GCC AAC TTC CTG
Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro Thr Ser Ala Asn Phe Leu
             20   30

ATC TGG CCC CCG TGC GTG GAG GTG AAA CGC TGC ACC GGC TGC TGC AAC
Ile Trp Pro Pro Cys Val Glu Val Lys Arg Cys Thr Gly Cys Cys Asn
                        40

ACG AGC AGT GTC AAG TGC CAG CCC TCC CGC GTC CAC CAC CGC AGC GTC
Thr Ser Ser Val Lys Cys Gln Pro Ser Arg Val His His Arg Ser Val
    50   60

AAG GTG GCC AAG GTG GAA TAC GTC AGG AAG AAG CCA AAA TTA AAA GAA
Lys Val Ala Lys Val Glu Tyr Val Arg Lys Lys Pro Lys Leu Lys Glu
                        70   80

GTC CAG GTG AGG TTA GAG GAG CAT TTG GAG TGC GCC TGC GCG ACC ACA
Val Gln Val Arg Leu Glu Glu His Leu Glu Cys Ala Cys Ala Thr Thr
                                       90

AGC CTG AAT CCG GAT TAT CGG GAA GAG GAC ACG GGA AGG CCT AGG GAG
Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp Thr Gly Arg Pro Arg Glu
        100   110

TCA GGT AAA AAA CGG AAA AGA AAA AGG TTA AAA CCC ACC
Ser Gly Lys Lys Arg Lys Arg Lys Arg Leu Lys Pro Thr
                    120

Translated Mol. Weight = 14307.74

Figure 2

SUBSTITUTE SHEET

```
TCT TTG GGT TCT TTG ACT ATC GCT GAA CCA GCT ATG ATC GCT GAA TGT
Ser Leu Gly Ser Leu Thr Ile Ala Glu Pro Ala Met Ile Ala Glu Cys
 1                                     10

AAG ACT AGA ACT GAA GTT TTC GAA ATC TCC AGA AGA TTG ATC GAC AGA
Lys Thr Arg Thr Glu Val Phe Glu Ile Ser Arg Arg Leu Ile Asp Arg
         20                                          30

ACT AAC GCT AAC TTC TTG GTT TGG CCA CCA TGT GTT GAA GTT CAA AGA
Thr Asn Ala Asn Phe Leu Val Trp Pro Pro Cys Val Glu Val Gln Arg
                         40

TGT TCT GGT TGT TGT AAC AAC AGA AAC GTT CAA TGT AGA CCA ACT CAA
Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln Cys Arg Pro Thr Gln
     50                                      60

GTT CAA TTG AGA CCA GTT CAA GTT AGA AAG ATC GAA ATC GTT AGA AAG
Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile Val Arg Lys
                 70                                          80

AAG CCA ATC TTC AAG AAG GCT ACT GTT ACT TTG GAA GAC CAC TTG GCT
Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu Ala
                                         90

TGT AAG TGT GAA ACT GTC GCC GCT GCC AGG CCA GTT ACT TAA TAG
Cys Lys Cys Glu Thr Val Ala Ala Ala Arg Pro Val Thr OC
             100
```

Translated Mol. Weight = 12296.37

Figure 3

Sequence of the Human Platelet-Derived
Growth Factor A-Chain Clone Uppsala-13-1

GAATTCCGTC CGCAAATATG CAGAATTACC GGCCGGGTCG CTCCTGAAGC CAGCGCGGGG

AGGCAGCGCG GCGGCGGCCA GCACCGGGAA CGCACCGAGG AAGAAGCCCA GCCCCCGCCC

TCCGCCCCTT CCGTCCCCAC CCCCATCCCG GCGGCCCAGG AGGCTCCCCG CGCTGGCGCG

CACTCCCTGT TTCTCCTCCT CCTGGCTGGC GCTGCCTGCC TCTCCGCACT CACTGCTCGC

CGGGCGCCGT CCGCCAGCTC CGTGCTCCCC GCGCCACCCT CCTCCGGGCC GCGCTCCCTA

AGGGATGGTA CTGATTTTCG CCGCCACAGG AGACCGGCTG GAGCGCCGCC CCGCGGCCTC

GCCTCTCCTC CGAGCAGCCA GCGCCTCGGG ACGCG ATG AGG ACC TTG GCT TGC
                                         Met Arg Thr Leu Ala Cys
                                          1

CTG CTG CTC CTC GGC TGC GGA TAC CTC GCC CAT GTT CTG GCC GAG GAA
Leu Leu Leu Leu Gly Cys Gly Tyr Leu Ala His Val Leu Ala Glu Glu
               10   20

GCC GAG ATC CCC CGC GAG GTG ATC GAG AGG CTG GCC CGC AGT CAG ATC
Ala Glu Ile Pro Arg Glu Val Ile Glu Arg Leu Ala Arg Ser Gln Ile
                             30

CAC AGC ATC CGG GAC CTC CAG CGA CTC CTG GAG ATA GAC TCC GTA GGG
His Ser Ile Arg Asp Leu Gln Arg Leu Leu Glu Ile Asp Ser Val Gly
           40   50

AGT GAG GAT TCT TTG GAC ACC AGC CTG AGA GCT CAC GGG GTC CAT GCC
Ser Glu Asp Ser Leu Asp Thr Ser Leu Arg Ala His Gly Val His Ala
                      60   70

ACT AAG CAT GTG CCC GAG AAG CGG CCC CTG CCC ATT CGG AGG AAG AGA
Thr Lys His Val Pro Glu Lys Arg Pro Leu Pro Ile Arg Arg Lys Arg
                               80

AGC ATC GAG GAA GCT GTC CCC GCT GTC TGC AAG ACC AGG ACG GTC ATT
Ser Ile Glu Glu Ala Val Pro Ala Val Cys Lys Thr Arg Thr Val Ile
                     90   100

TAC GAG ATT CCT CGG AGT CAG GTC GAC CCC ACG TCC GCC AAC TTC CTG
Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro Thr Ser Ala Asn Phe Leu
                            110

ATC TGG CCC CCG TGC GTG GAG GTG AAA CGC TGC ACC GGC TGC TGC AAC
Ile Trp Pro Pro Cys Val Glu Val Lys Arg Cys Thr Gly Cys Cys Asn
      120   130

Figure 4 (Sheet 1 of 2)

```
ACG AGC AGT GTC AAG TGC CAG CCC TCC CGC GTC CAC CAC CGC AGC GTC
Thr Ser Ser Val Lys Cys Gln Pro Ser Arg Val His His Arg Ser Val
                140                                             150

AAG GTG GCC AAG GTG GAA TAC GTC AGG AAG AAG CCA AAA TTA AAA GAA
Lys Val Ala Lys Val Glu Tyr Val Arg Lys Lys Pro Lys Leu Lys Glu
                                    160

GTC CAG GTG AGG TTA GAG GAG CAT TTG GAG TGC GCC TGC GCG ACC ACA
Val Gln Val Arg Leu Glu Glu His Leu Glu Cys Ala Cys Ala Thr Thr
                170                                     180

AGC CTG AAT CCG GAT TAT CGG GAA GAG GAC ACG GAT GTG AGG TGA
Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp Thr Asp Val Arg OP
                                190
```

GGATGAGCCG CAGCCCTTTC CTGGGACATG GATGTACATG GCGTGTTACA TTCCTGAACC

TACTATGTAC GGTGCTTTAT TGCCAGTGTG CGGTCTTTGT TCTCCTCCGT GAAAAACTGT

GTCCGAGAAC ACTCGGGAGA ACAAAGAGAC AGTGCACATT TGTTTAATGT GACATCAAAG

CAAGTATTGT AGCACTCGGT GAAGCAGTAA GAAGCTTCCT TGTCAAAAAG AGAGAGAGAG

AAAAGAAAAA AAAAGGAATT C

Translated Mol. Weight = 22256.17

Figure 4 (Sheet 2 of 2)

Sequence of the Human Platelet-Derived
Growth Factor A-Chain Clone Uppsala-D1

GAATTCCGTC CGCAAATATG CAGAATTACC GGCCGGGTCG CTCCTGAAGC CAGCGCGGGG

AGGCAGCGCG GCGGCGGCCA GCACCGGGAA CGCACCGAGG AAGAAGCCCA GCCCCCGCCC

TCCGCCCCTT CCGTCCCCAC CCCCATCCCG GCGGCCCAGG AGGCTCCCCG CGCTGGCGCG

CACTCCCTGT TTCTCCTCCT CCTGGCTGGC GCTGCCTGCC TCTCCGCACT CACTGCTCGC

CGGGCGCCGT CCGCCAGCTC CGTGCTCCCC GCGCCACCCT CCTCCGGGCC GCGCTCCCTA

AGGGATGGTA CTGATTTTCG CCGCCACAGG AGACCGGCTG GAGCGCCGCC CCGCGGCCTC

GCCTCTCCTC CGAGCAGCCA GCGCCTCGGG ACGCG ATG AGG ACC TTG GCT TGC
                                        Met Arg Thr Leu Ala Cys
                                         1

CTG CTG CTC CTC GGC TGC GGA TAC CTC GCC CAT GTT CTG GCC GAG GAA
Leu Leu Leu Leu Gly Cys Gly Tyr Leu Ala His Val Leu Ala Glu Glu
              10   20

GCC GAG ATC CCC CGC GAG GTG ATC GAG AGG CTG GCC CGC AGT CAG ATC
Ala Glu Ile Pro Arg Glu Val Ile Glu Arg Leu Ala Arg Ser Gln Ile
                          30

CAC AGC ATC CGG GAC CTC CAG CGA CTC CTG GAG ATA GAC TCC GTA GGG
His Ser Ile Arg Asp Leu Gln Arg Leu Leu Glu Ile Asp Ser Val Gly
    40   50

AGT GAG GAT TCT TTG GAC ACC AGC CTG AGA GCT CAC GGG GTC CAT GCC
Ser Glu Asp Ser Leu Asp Thr Ser Leu Arg Ala His Gly Val His Ala
                          60   70

ACT AAG CAT GTG CCC GAG AAG CGG CCC CTG CCC ATT CGG AGG AAG AGA
Thr Lys His Val Pro Glu Lys Arg Pro Leu Pro Ile Arg Arg Lys Arg
                                          80

AGC ATC GAG GAA GCT GTC CCC GCT GTC TGC AAG ACC AGG ACG GTC ATT
Ser Ile Glu Glu Ala Val Pro Ala Val Cys Lys Thr Arg Thr Val Ile
                  90   100

TAC GAG ATT CCT CGG AGT CAG GTC GAC CCC ACG TCC GCC AAC TTC CTG
Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro Thr Ser Ala Asn Phe Leu
                          110

ATC TGG CCC CCG TGC GTG GAG GTG AAA CGC TGC ACC GGC TGC TGC AAC
Ile Trp Pro Pro Cys Val Glu Val Lys Arg Cys Thr Gly Cys Cys Asn
    120   130

ACG AGC AGT GTC AAG TGC CAG CCC TCC CGC GTC CAC CAC CGC AGC GTC
Thr Ser Ser Val Lys Cys Gln Pro Ser Arg Val His His Arg Ser Val
                  140   150

Figure 5 (Sheet 1 of 2)

```
AAG GTG GCC AAG GTG GAA TAC GTC AGG AAG AAG CCA AAA TTA AAA GAA
Lys Val Ala Lys Val Glu Tyr Val Arg Lys Lys Pro Lys Leu Lys Glu
                                         160

GTC CAG GTG AGG TTA GAG GAG CAT TTG GAG TGC GCC TGC GCG ACC ACA
Val Gln Val Arg Leu Glu Glu His Leu Glu Cys Ala Cys Ala Thr Thr
                170                                         180

AGC CTG AAT CCG GAT TAT CGG GAA GAG GAC ACG GGA AGG CCT AGG GAG
Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp Thr Gly Arg Pro Arg Glu
                                 190

TCA GGT AAA AAA CGG AAA AGA AAA AGG TTA AAA CCC ACC TAA AGCAGCCA
Ser Gly Lys Lys Arg Lys Arg Lys Arg Leu Lys Pro Thr OC
    200                                           210

ACCAGATGTG AGGTGAGGAT GAGCCGCAGC CCTTTCCTGG GACATGGATG TACATGGCGT

GTTACATTCC TGAACCTACT ATGTACGGTG CTTTATTGCC AGTGTGCGGT CTTTGTTCTC

CTCCGTGAAA AACTGTGTCC GAGAACACTC GGGAGAACAA AGAGACAGTG CACATTTGTT

TAATGTGACA TCAAAGCAAG TATTGTAGCA CTCGGTGAAG CAGTAAGAAG CTTCCTTGTC

AAAAAGAGAG AGAGAGAAAA GAAAAAAAAA GGAATTC
```

Translated Mol. Weight = 24046.60

Figure 5 (Sheet 2 of 2)
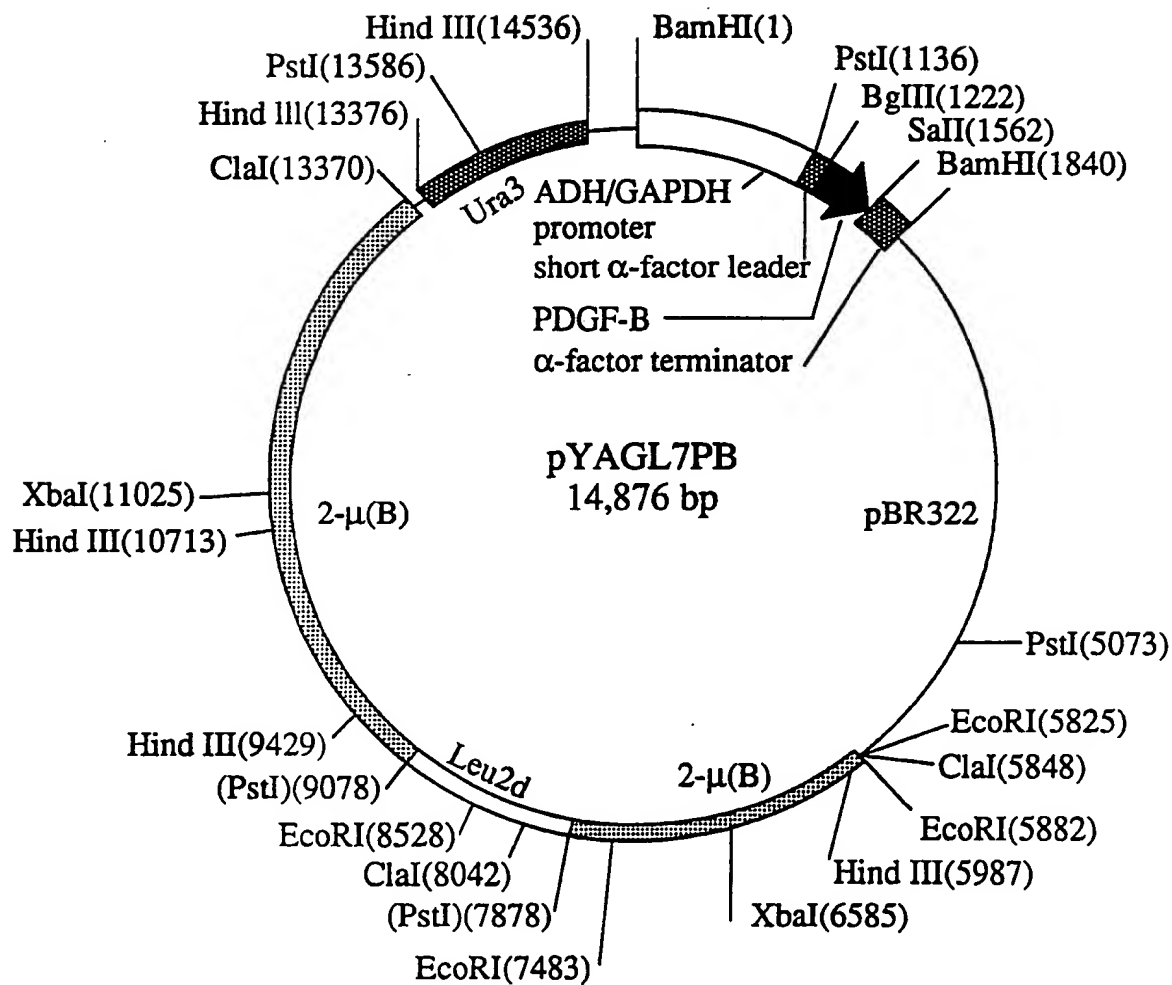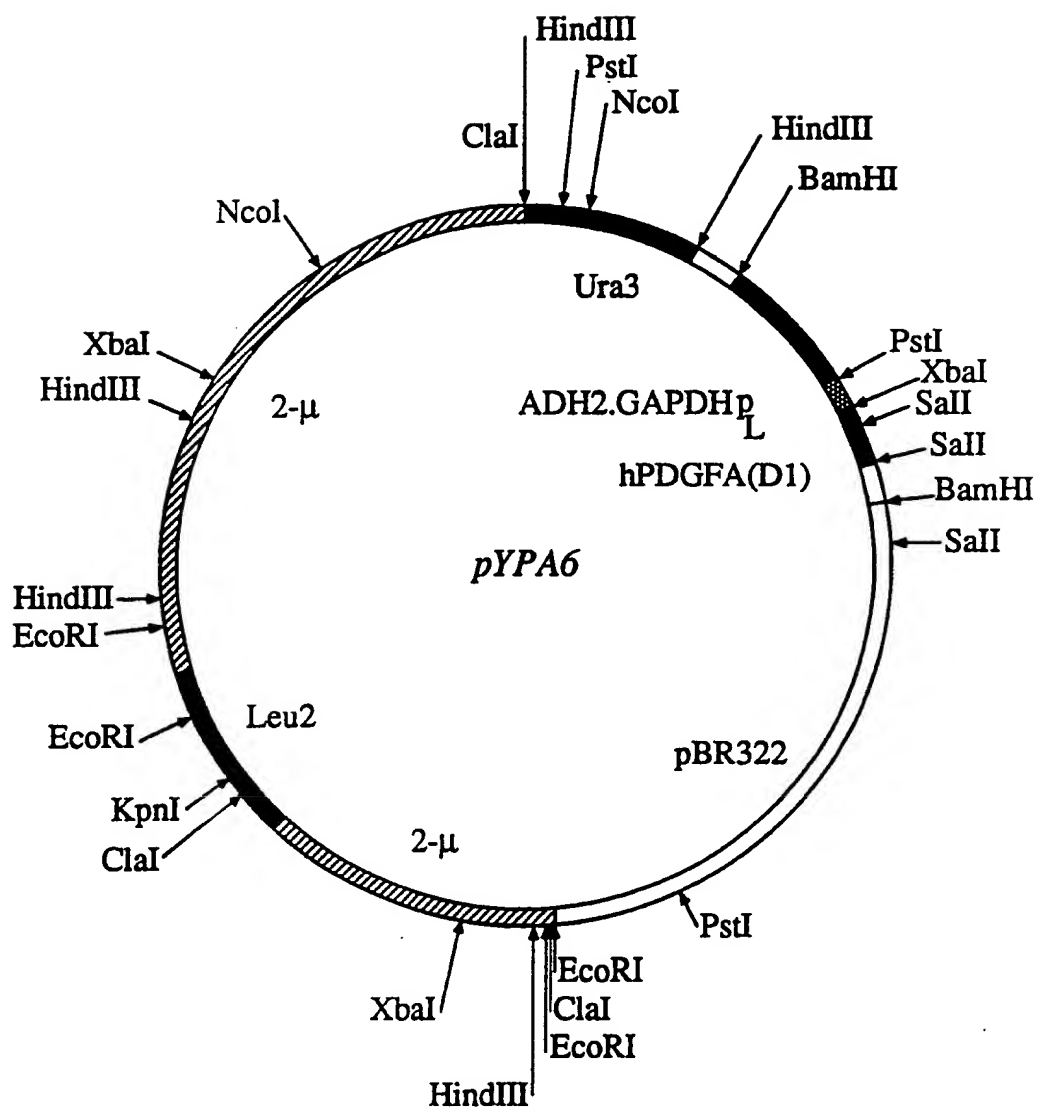
Figure 6

9/16

Figure 7

HindIII
PstI
NcoI
ClaI
HindIII
BamHI
Ncol
XbaI
HindIII
2μ
Ura3
ADH2.GAPDHp
L
hPDGFA(13)
pYPA134
PstI
XbaI
SaII
SaII
BamHI
SaII
HindIII
EcoRI
EcoRI
Leu2
KpnI
ClaI
2μ
pBR322
PstI
XbaI
EcoRI
ClaI
EcoRI
HindIII
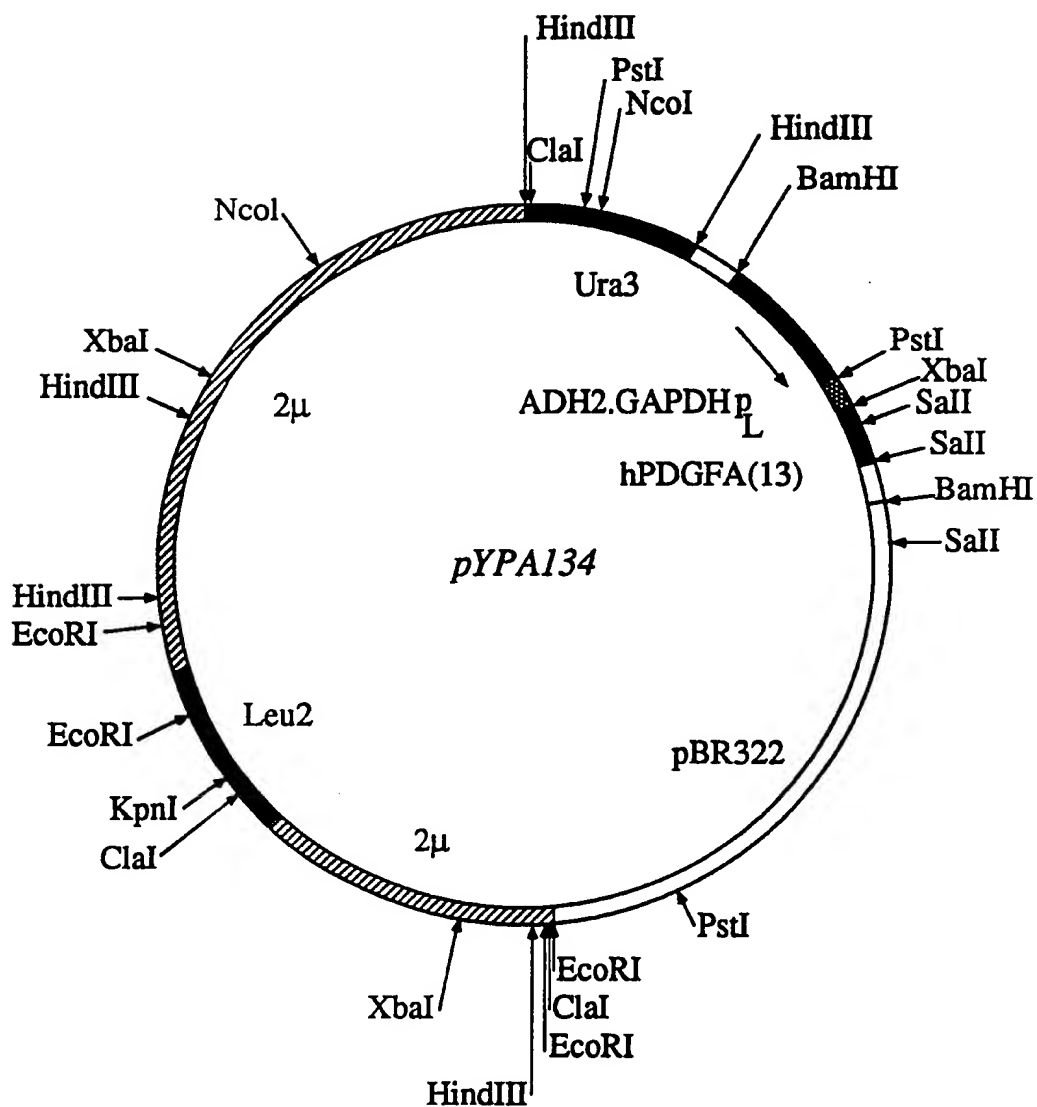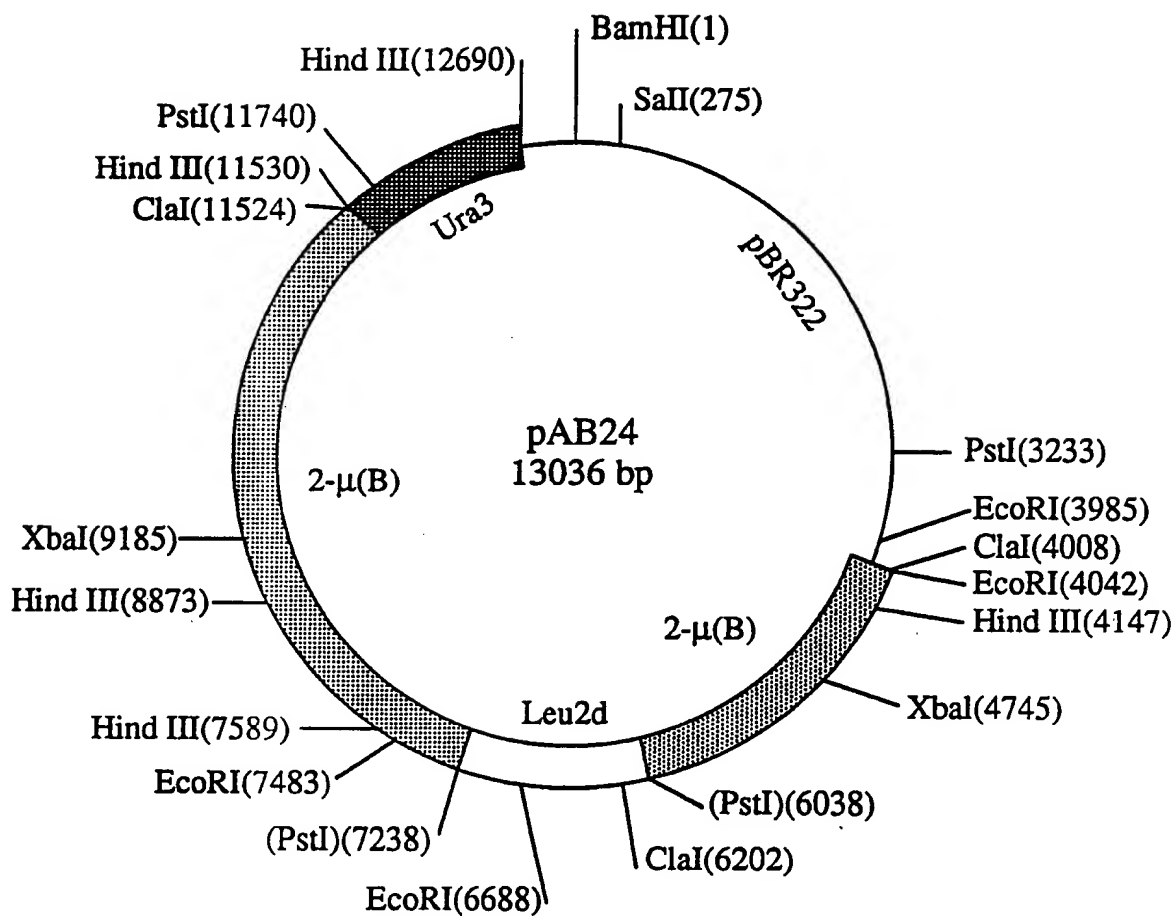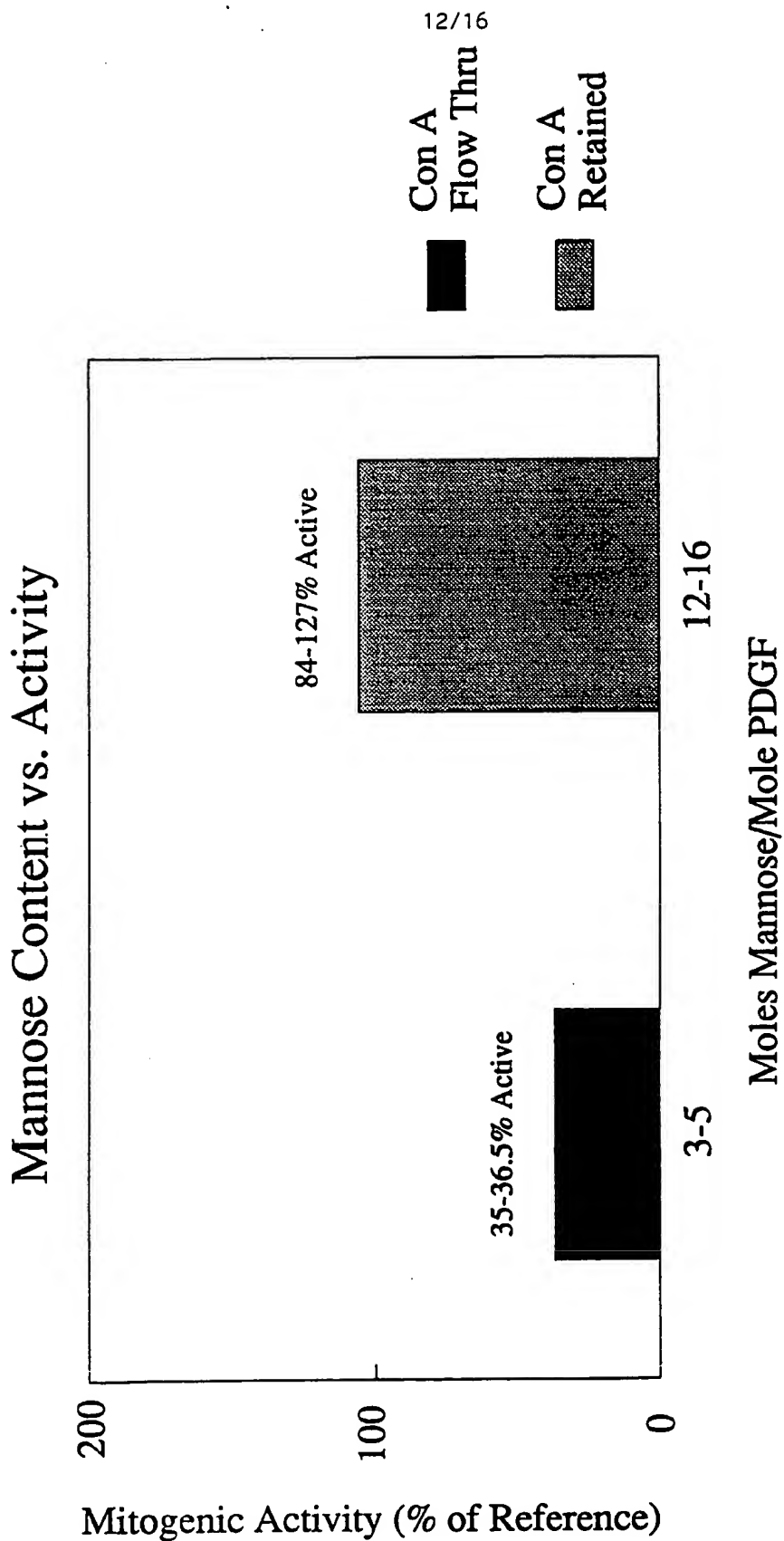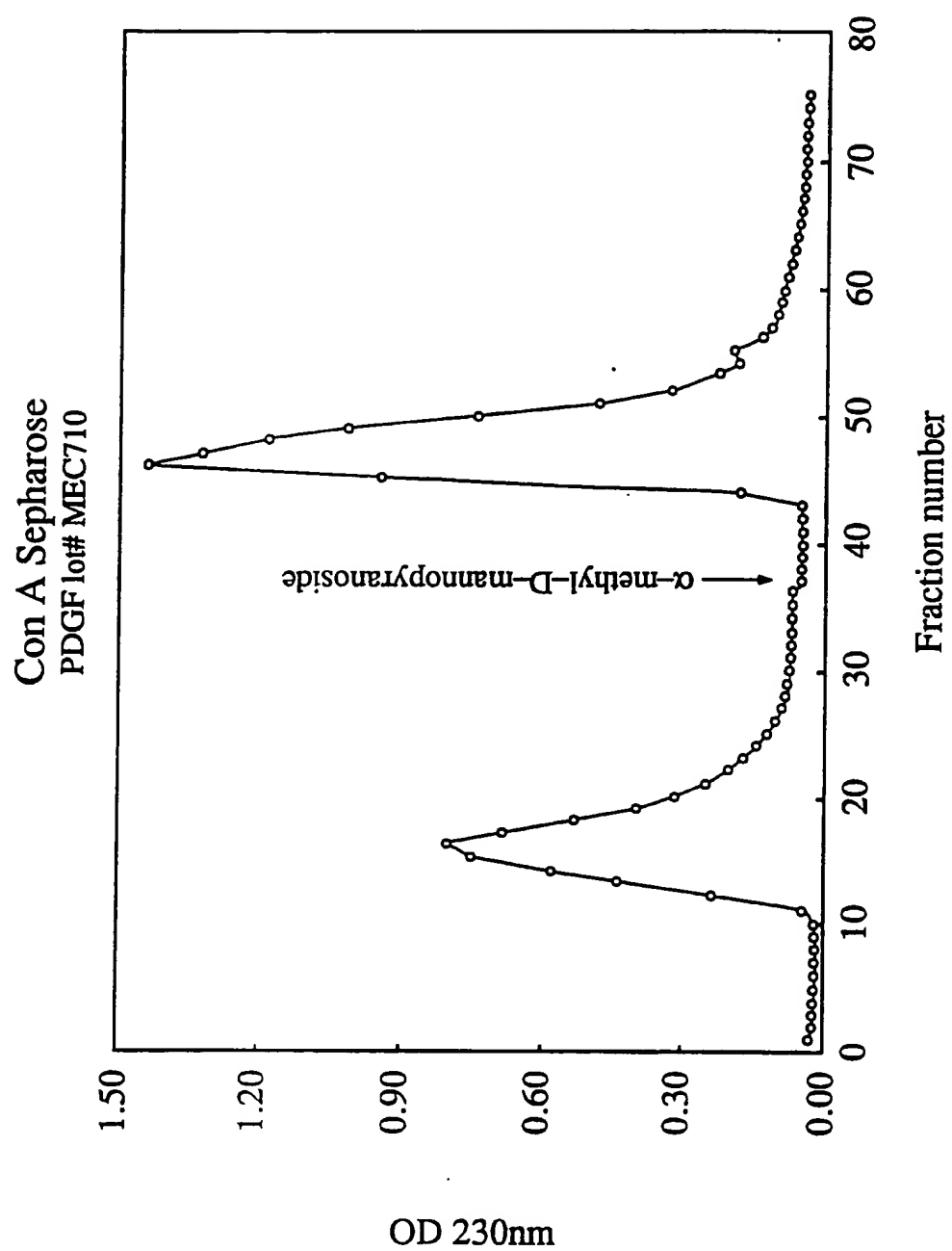
Figure 8

Figure 9
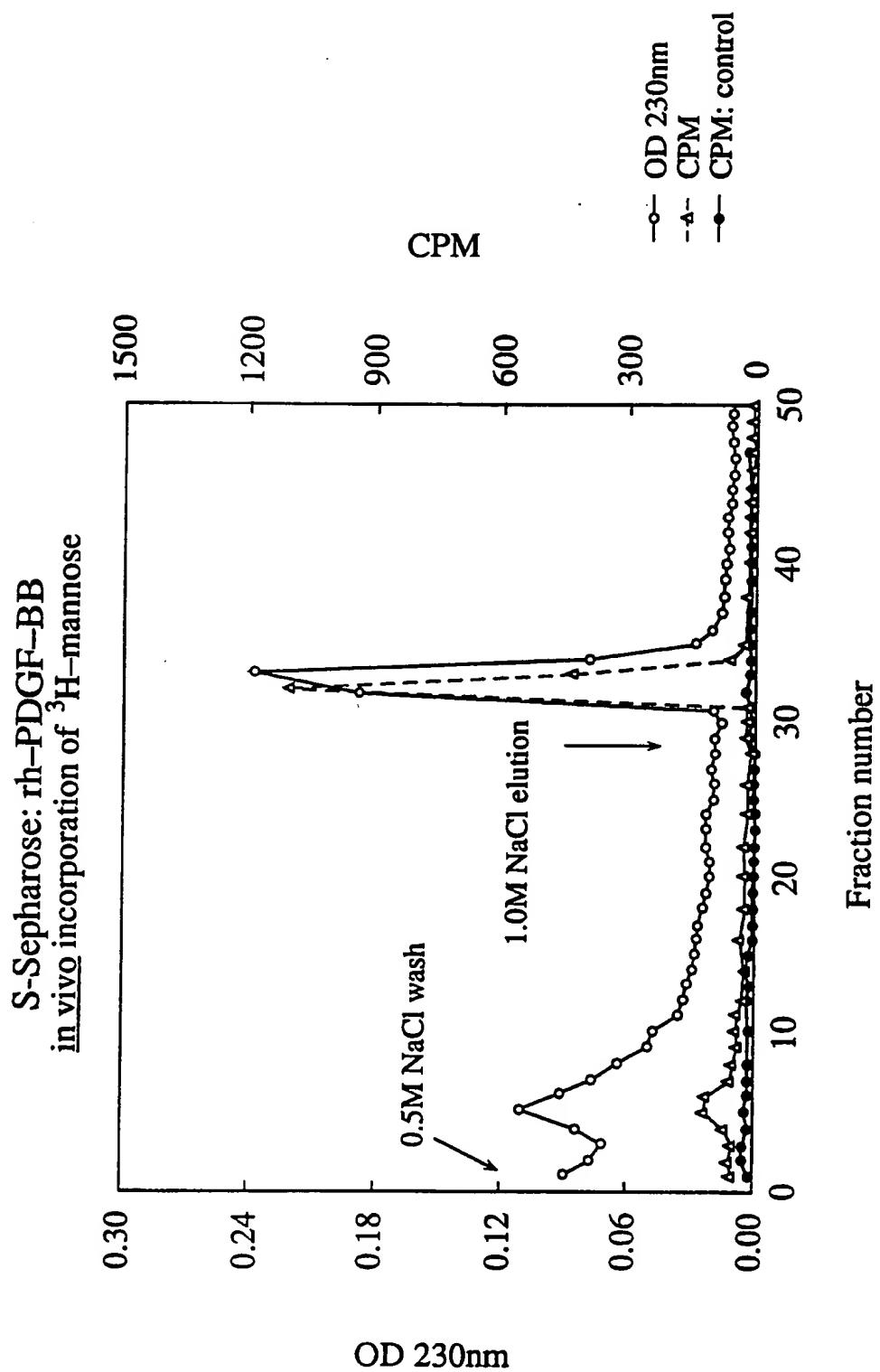
Figure 10

Figure 11

Figure 12

Sequence of SV40 early pr moter, polylinker, and SV40
poly A addition region from pSV7d

| 1/2 PVU11 site SV40   early   promoter --->
CTGTGGAATG TGTGTCAGTT AGGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT
GACACCTTAC ACACAGTCAA TCCCACACCT TTCAGGGGTC CGAGGGGTCG TCCGTCTTCA

 61 ATGCAAAGCA TGCATCTCAA TTAGTCAGCA AGGAAAGTCC CCAGGCTCCC CAGCAGGCAG
    TACGTTTCGT ACGTAGAGTT AATCAGTCGT TCCTTTCAGG GGTCCGAGGG GTCGTCCGTC

    68 SPH1

121 AAGTATGCAA AGCATGCATC TCAATTAGTC AGCAACCATA GTCCCGCCCC TAACTCCGCC
    TTCATACGTT TCGTACGTAG AGTTAATCAG TCGTTGGTAT CAGGGCGGGG ATTGAGGCGG

    132 SPH1

181 CATCCCGCCC CTAACTCCGC CCAGTTCCGC CCATTCTCCG CCCCATGGCT GACTAATTTT
    GTAGGGCGGG GATTGAGGCG GGTCAAGGCG GGTAAGAGGC GGGGTACCGA CTGATTAAAA

    223 NCO1,

241 TTTTATTTAT GCAGAGGCCG AGGCCGCCTC GGCCTCTGAG CTATTCCAGA AGTAGTGAAG
    AAAATAAATA CGTCTCCGGC TCCGGCGGAG CCGGAGACTC GATAAGGTCT TCATCACTTC

    263 BGL1

                   |-polylinker--->   OC OP OP
301 AGGCTTTTTT GGAGGAGATC GAATTCCCGG GTCTAGAGGA TCCGTCGACC TAGATAAGTA
    TCCGAAAAAA CCTCCTCTAG CTTAAGGGCC CAGATCTCCT AGGCAGCTGG ATCTATTCAT

    321 ECOR1,   326 SMA1 XMA1, 332 XBA1, 338 BAMH1, 344 SAL1,

361 ATGATCATAA TCAGCCATAT CACATCTGTA GAGGTTTTAC TTGCTTTAAA AAACCTCCCA
    TACTAGTATT AGTCGGTATA GTGTAGACAT CTCCAAAATG AACGAAATTT TTTGGAGGGT

    362 BCL1, 405 DRA1,

421 CACCTCCCCC TGAACCTGAA ACATAAAATG AATGCAATTG TTGTTGTTAA CTTGTTTATT
    GTGGAGGGGG ACTTGGACTT TGTATTTTAC TTACGTTAAC AACAACAATT GAACAAATAA

    466 HPA1

481 GCAGCTTATA ATGGTTACAA ATAAAGCAAT AGCATCACAA ATTTCACAAA TAAAGCATTT
    CGTCGAATAT TACCAATGTT TATTTCGTTA TCGTAGTGTT TAAAGTGTTT ATTTCGTAAA

                    End of SV40----->|| -pBR322 (pos. 4210)->
541 TTTTCACTGC ATTCTAGTTG TGGTTTGTCC AAACTCATCC GCTCATGAGA CAATAACCCT
   AAAAGTGACG TAAGATCAAC ACCAAACAGG TTTGAGTAGG CGAGTACTCT GTTATTGGGA

Figure 13

SUBSTITUTE SHEET
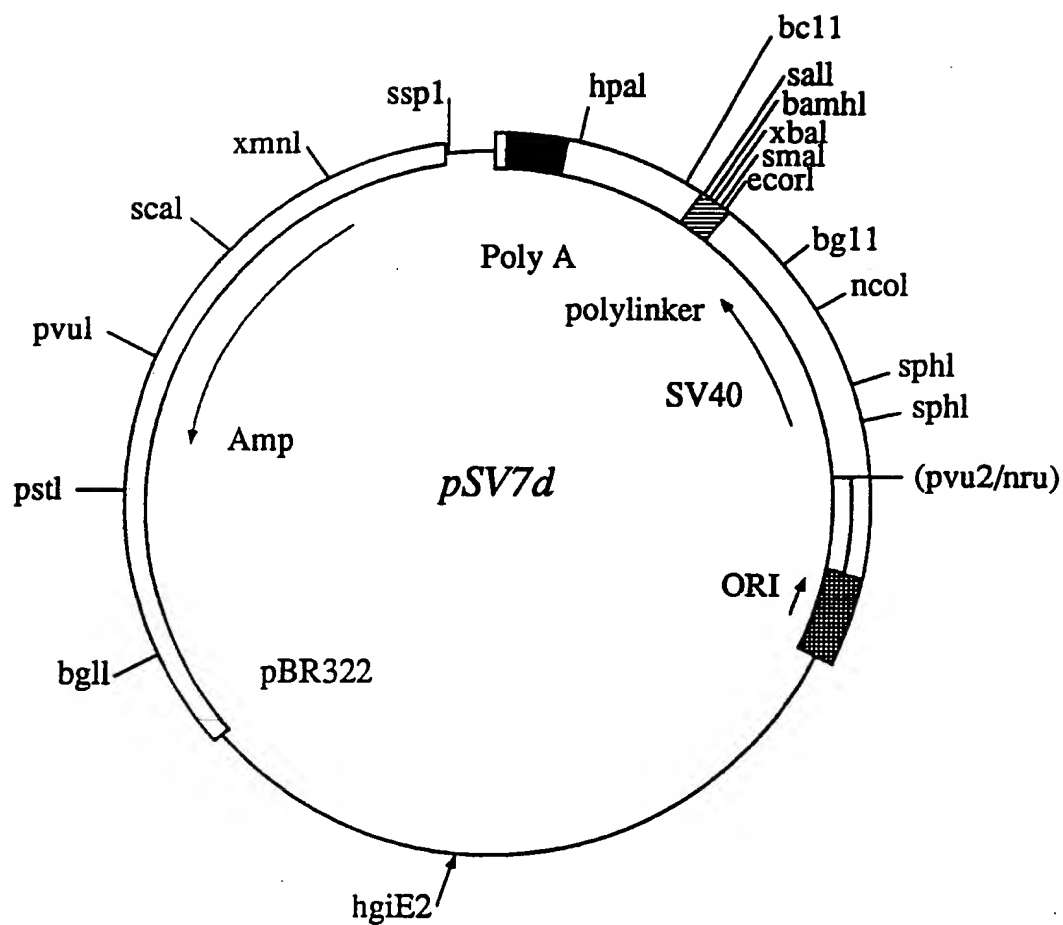
Figure 14

INTERNATIONAL SEARCH REPORT

International Applic... on No. PCT/US91/02766

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 3/12, 3/28
U.S. CL.: 530/350; 435/172.3, 69.9, 69.4

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/350 435/172.3, 69.9, 69.4

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Dialog (files 5, 399, 155, 154, 357, 72)
Automated Patent System (File USPAT, 1971-1991)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with Indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Virology, Volume 164, issued 1988, KLEIN ET AL., "Highly glycosylated PDGF-like Molecule Secreted by Simian Sarcoma Virus-Transformed Cell", pages 403-410 (See entire document).	1-5
Y	US, A, 4,845,075 (MURRAY ET AL.) 04 July 1989, see entire document.	6-24
Y	US, A, 4,766,073 (MURRAY ET AL.) 23 August 1988, see enitre document.	6-24
Y	US, A, 4,849,407 (MURRAY ET AL.) 18 July 1989, see entire document.	6-24

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family.

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 June 1991	18 JUL 1991
International Searching Authority ISA/US	Signature of Authorized Officer Lynette F. Smith (vsh)

Form PCT/ISA/210 (second sheet) (Rev. 11-87)